LAB-SCALE OPTIMISATION OF KEFIR BEVERAGE PRODUCTION FROM MASS-CULTURED AND FREEZE-DRIED KEFIR GRAINS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entirety or in part, been submitted at any other university for a degree.

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ABSTRACT

Kefir is a fermented dairy beverage resulting from the fermentation of milk with reusable Kefir grains. The grains consist of a complex combination of lactic acid bacteria and yeasts in a symbiotic relationship, embedded in a polysaccharide matrix called kefiran. Various problems are experienced during the commercialisation of the ready-made Kefir beverage and, therefore, it is more advantageous to market the grains, enabling the consumer to produce the beverage at home. Kefir grains could be mass-cultured and then preserved by lyohilisation for successful long-term storage and easy distribution during commercialisation. The microbial balance of the Kefir grains changes during both mass-culturing and freeze-drying, which will have an influence on the sensory properties of the Kefir beverage produced. The aim of this study was the optimisation of the production of Kefir from mass-cultured grains and from freeze-dried mass-cultured grains respectively. The sensory characteristics of the fermented beverages produced from these mass-cultured and preserved grains were determined.

Mass-cultured Kefir grains were activated and Kefir produced using nine methods with different activation times and temperatures, different grain:milk ratios (36, 72 and 108 g grains.l^{-1}) and with different heat-treated milks (pasteurised, double pasteurised and UHT). The best Kefir beverage was produced by activation of the grains at 22°C for two successive 24 h incubation periods, followed by Kefir production at 22°C for 18 h and a maturation period at 18°C for 6 h. The milk was replaced before every incubation period, excluding the maturation period, and the fermentation vessel was swirled five times at the start of fermentation and after 18 h. This method resulted in a sour beverage with a thick consistency and the characteristic effervescence and flavour of Kefir. The optimal grain:milk ratio was identified as 36 g grains.l^{-1} and the best heat-treated milks for the production of Kefir beverage were UHT and double pasteurised milk.

Mass-cultured Kefir grains were freeze-dried for 1, 2, 3 and 6 d and the moisture loss determined. Freeze-dried grains were rehydrated for 1, 2, 6, 12 and 18 h to determine the optimal rehydration time. A sensory analysis was performed to compare the properties of Kefir produced from mass-cultured grains (MC), freeze-dried mass-cultured grains that were rehydrated and activated (FDRA) and

activated mass-cultured grains that were freeze-dried and rehydrated (AFDR). The chemical compositions of mass-cultured grains (MC), mass-cultured, freeze-dried grains (MCFD), mass-cultured, freeze-dried grains that were rehydrated and activated (FDRA) and activated mass-cultured grains that were freeze-dried and rehydrated (AFDR), were also investigated. The optimum time to freeze-dry grains was 2 d and to rehydrate freeze-dried grains was 1 h. The sensory analysis indicated that Kefir beverages prepared from FDRA and AFDR grains did not differ significantly and were less fermented than Kefir produced from MC grains.

It was concluded that Kefir with excellent sensory characteristics can be produced from mass-cultured grains. Freeze-drying is a better method to preserve Kefir grains than freezing due to mass loss during freezing and easier distribution and storage of freeze-dried grains. The supplementation of freeze-dried grains with additional lactic acid bacteria and yeast isolates should be investigated.

UITTREKSEL

Kefir is 'n gefermenteerde suiwelproduk wat geproduseer word deur die fermentasie van melk met herbruikbare Kefirkorrels. Die korrels bestaan uit 'n komplekse kombinasie van melksuurbakterië en giste en is ingebed in 'n polisakkaried matriks genaamd kefiran. Verskeie probleme word ondervind met die kommersialisering van die klaar voorbereide Kefirdrankie en dit is meer voordelig om die korrels te bemark. Dit sal die verbruiker daartoe in staat stel om self Kefir tuis te produseer. Kefirkorrels kan in massa gekweek word en dan gevriesdroog word om langtermyn storing en verspreiding te vergemaklik tydens kommersialisering. Die spesifieke mikrobiese balans van die Kefirkorrels word tydens massakweking en vriesdroging versteur. Dus sal hierdie twee prosesse 'n invloed hê op die sensoriese eienskappe van die Kefir drankie geproduseer. Die doel van hierdie studie was die optimisering van die produksie van Kefir vanaf massagekweekte korrels en gevriesdroogde massagekweekte korrels. Die sensoriese karakteristieke van die Kefir geproduseer met hierdie korrels is ondersoek.

Massagekweekte Kefirkorrels is geaktifeer en Kefir is geproduseer met nege verskillende metodes met variasies in die tyd en temperatuur kombinasies, verskillende korrel:melk verhoudings (36, 72 en 108 g korrels.l^{-1}) en verskillende hittebehandelde melke (gepasteuriseerd, dubbel gepasteuriseer en UHT). Die beste Kefirdrankie is geproduseer deur die aktivering van die korrels by 22°C vir twee 24 h inkubasieperiodes, gevolg deur Kefir produksie by 22°C vir 18 uur en 'n verouderingsperiode by 18°C vir 6 h. Die melk was voor elke inkubasieperiode vervang, uitsluitende die verouderingsperiode. Die fermentasie houer is vyf maal gedraai aan die begin van fermentasie en na 12 h. Hierdie metode het gelei tot 'n drankie wat suur was met 'n dik konsistensie en die karakteristieke vonkeling en geur van Kefir. Die optimale korrel:melk ratio is geidentifiseer as 36 g korrels.l^{-1} en die verkieslike hittebehandelde melke is dubbel gepasteuriseerde en UHT melk.

Massagekweekte Kefirkorrels was vir 1, 2, 3 en 6 dae gevriesdroog en die massaverlies is bepaal. Gevriesdroog korrels is gerehidreer vir 1, 2, 6, 12 en 18 h om die optimale rehidrasietyd te bepaal. 'n Sensoriese analise is uitgevoer om die eienskappe te vergelyk van Kefir geproduseer van massagekweekte korrels (MC), gevriesdroogde massagekweekte korrels wat gerehidreer en geaktiveer is (FDRA) en geaktiveerde massagekweekte korrels wat gevriesdroog en gerehidreed is

(AFDR). Die chemiese samestelling van massagekweekte korrels (MC), massagekweekte, gevriesdroogde korrels (MCFD), massagekweekte, gevriesdroogde korrels wat gerehidreer en geaktiveer is (FDRA) en geaktiveerde massagekweekte korrels wat gevriesdroog en gerehidreer is (AFDR), is bepaal. Die optimum tydperk vir vriesdroging van korrels was 2 d en vir rehidrasie van gevriesdroogde korrels was 1 h. Die sensoriese analise het aangedui dat Kefir wat van FDRA en AFDR korrels geproduseer is, nie betekenisvol van mekaar verskil het nie, maar minder gefermenteerd was as Kefir wat van MC korrels geproduseer is.

Die gevolgtrekking is gemaak dat 'n Kefirdrankie met uitstekende eienskappe geproduseer kan word met massagekweekte korrels. Vriesdroging is 'n beter metode as bevriesing om Kefirkorrels te preserveer a.g.v die verlies van massa tydens bevriesing en die vergemakliking van vervoer en verspreiding van gevriesdroogde korrels. Die aanvulling van gevriesdroogde korrels met addisionele melksuurbakterieë en giste moet nog ondersoek word.

CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

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"He who learns but does not think, is lost.
He who thinks but does not learn is in great danger."

- Confucius (551 - 479 b.c.) -

CHAPTER 1

INTRODUCTION

Milk is a highly nutritious substance, but its consumption is low amongst black South Africans due the high occurrence of lactose intolerance in this population group (MacIntyre *et al.*, 2002). Lactose intolerant people are, however, able to consume fermented milk products. Maas, a fermented milk beverage that is traditionally made by allowing unpasteurised milk to sour, is the most popular fermented milk in South Africa (Keller & Jordaan, 1990). Due to legislation, stipulating that raw milk may not be sold unless it is to be further processed, the low-income Black urban communities are not able to make traditional Maas as the production of Maas is not considered to be "further processing" (Anon., 1997). Maas produced from pasteurised milk is commercially available, but it is expensive and a poor equivalent to the traditional Maas since it contains colourants and preservatives and has different sensory characteristics (Berry, 1999; Van Wyk *et al.*, 2002). Therefore, there exists need for a fermented dairy product that has sensory characteristics comparable to that of traditional Maas, is low in cost, nutritious and safe to consume. A product that fulfils these requirements is Kefir (Van Wyk *et al.*, 2002).

Kefir is a self-carbonated, fermented dairy beverage with a yeasty and sour flavour and a refreshing effervescence (Özer & Özer, 2000). It has a taste comparable to that of traditional Maas and is easy to prepare at home (Garrote *et al.*, 1998; Van Wyk *et al.*, 2002). It is produced by fermenting milk with reusable Kefir grains that, if handled correctly, will remain active for many years (Steinkraus, 1996). The consumer will only have to purchase the grains once and, if handled correctly, the expense of making Kefir thereafter will merely be that of the milk (Van Wyk *et al.*, 2002).

Kefir grains are moist, white to yellow coloured and irregular in shape with a diameter of 0.3 – 3.0 cm. They are a stable conglomerate of lactic acid bacteria and yeasts imbedded in and held together by an insoluble polysaccharide material called kefiran (Steinkraus, 1996). The microbial and chemical composition of Kefir

grains vary depending on their origin and methods of cultivation (Libudzisz & Piatkiewicz, 1990; Kurmann *et al.*, 1992; Özer & Özer, 2000; Garrote *et al.*, 2001).

Various problems have been encountered with the commercialisation of ready prepared Kefir. Secondary alcohol fermentation by the yeasts leads to blowing of containers and leakage due to internal pressure (Kwak *et al.*, 1996). Difficulty is also experienced to produce a Kefir with consistent characteristics and quality due to the changing balance and activity of the microbes (Kemp, 1984; Kuo & Lin, 1999). Due to these problems and to minimise the cost of Kefir for the low-income consumer, it will be preferable to commercialise the Kefir grains in South Africa for household production of Kefir.

Kefir grains increase in size and number during continuous cultivation in milk (Marshall & Cole, 1985), but under normal circumstances their growth is extremely slow (Saloff-Coste, 1996). Schoevers & Britz (2003) developed a method for the rapid mass-culturing of Kefir grains. However, the microbial population of the Kefir grains changes during mass-culturing, which will have an influence on the sensory properties of the final Kefir beverage produced (Kuo & Lin, 1999; Witthuhn *et al.*, 2004).

Freeze-drying is the most effective method to preserve Kefir grains for long-term storage and easy distribution during commercialisation (Brialy *et al.*, 1995; Cilliers, 2001). However, difficulties are encountered with the survival of the yeast component of the grains during freeze-drying and between 80 and 90% of the yeasts may be lost (Duitschaever, 1989; Marshall, 1993; Liu *et al.*, 1999). Freeze-dried grains consist almost entirely of lactic acid bacteria leading to a product that lacks the effervescent characteristic and ethanol content of the traditional Kefir beverage (Marshall & Cole, 1985; Liu *et al.*, 1999).

Freeze-dried mass-cultured Kefir grains could be marketed successfully to the low-income black consumer market in South Africa. However, both mass-culturing and freeze-drying have an influence on the specific microbial population of the grains, which will determine the sensory characteristics. The sensory properties of such grains have not yet been investigated. It is, therefore, necessary to study the sensory characteristics of Kefir produced from mass-cultured and freeze-dried grains and to optimise the methods of activation of the grains and production of the Kefir to achieve a beverage with acceptable sensory characteristics.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Kefir is an ancient traditional fermented milk that originated in the Caucasian mountains (Kemp, 1984; Duitschaever, 1989; Garrote *et al.*, 2000). It is an acidic, mildly alcoholic, effervescent milk product and is obtained by the fermentative activity of so-called Kefir grains (Duitschaever, 1989; Garrote *et al.*, 2000). Kefir is often referred to as "the champagne of cultured dairy products", due to its effervescent characteristics (Merin & Rosenthal, 1986). It is also known as kepi, kephir, kiaphur, kefyr, képhir, kéfer, knapon and kippe. The word "Kefir" is thought to be derived from the Turkish word *kef* or *kefy* meaning "pleasant taste" (Kurmann *et al.*, 1992).

For centuries, the manufacture of Kefir was known only to members of the Ossete and Karabbiner tribes, indigenous to the Caucasian region (Duitschaever, 1989). During the second half of the nineteenth century knowledge of the production of Kefir spread and the product became popular in Eastern and Central Europe. It is currently consumed in various countries, including the countries of the former Soviet Union, Poland, the Czech Republic, Scandinavia, Hungary, Germany and Sweden (Kurmann *et al.*, 1992; Oberman & Libudzisz, 1998). The starters and production methods used in these countries vary, leading to Kefir beverages with different characteristics (Oberman & Libudzisz, 1998).

Kefir is reported to have a pH of approximately 4 – 4.4 with a lactic acid content of 0.68 - 1.5% (Duitschaever *et al.*, 1987; Steinkraus, 1996). The ethanol content may range between 0.1 and 2.5%, while the CO_2 content is between 0.08 and 0.2% (Kurmann *et al.*, 1992; Oberman & Libudzisz, 1998; Muir *et al.*, 1999). A good quality Kefir typically contains 10^9 lactic acid streptococci, 10^7 - 10^8 thermophilic lactobacilli, 10^4 - 10^5 yeasts and 10^4 - 10^5 acetic acid bacteria per ml (Koroleva, 1988b).

Kefir is considered exceptionally nutritious and in Russian hospitals it is often included in the diets of patients suffering from intestinal diseases, metabolic

disorders, arteriosclerosis and allergic diseases (Koroleva, 1988b; Garrote *et al.*, 2000). Kefir has been recommended for inclusion in special dietetic programs and has been used for the treatment of tuberculosis, various cancers and gastrointestinal disorders when modern medicinal treatment was not available (Kurmann *et al.*, 1992; Saloff-Coste, 1996).

B. THE KEFIR GRAIN

The starter culture used to produce Kefir is what differentiates it from other fermented milks. These milks are produced by the metabolic activity of evenly distributed microbes, while Kefir is made from a mixture of microbes sustained in Kefir grains. These grains can be recovered after fermentation and used to inoculate a new batch of milk (Marshall *et al.*, 1984). There is no written record of the first Kefir grains and it is thought to have developed by accident (Kemp, 1984).

General characteristics

Kefir grains are white to yellow and of variable size with a diameter that ranges between 0.3 and 3.0 cm (Kemp, 1984; Duitschaver, 1989; Marshall, 1993; Garrote *et al.*, 2001). The grains are composed of particles of clotted milk, a complex microbial population that consists mainly of yeasts and lactic acid bacteria, and products of their autolysis (Marshall & Cole, 1985; Oberman & Libudzisz, 1998). The grains first form as tiny granules and gradually grow during incubation in milk (Steinkraus, 1996). They are moist, elastic and have a particular characteristic smell (Libudzisz & Piatkiewicz, 1990; Steinkraus, 1996). Kefir grains are characterised by an irregular form, folded or uneven surface and has a shape similar to that of cauliflower florets, cooked rice or popped corn (Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Oberman & Libudzisz, 1998; Garrote *et al.*, 2001). They appear to arise from the curling of flat, sheet-like structures with a subsequent folding and refolding into a globular structure (Marshall, 1993).

Kefir grains from different sources differ in their composition. Grains that originated from Russia, Yugoslavia and Bulgaria constituted approximately 90% water, 3.2% protein, 0.3% lipids, 5.8% non-nitrogenous soluble substances and 0.7% ash (Ottogalli *et al.*, 1973). Kefir grains from Argentina were found to comprise of between 79 and 83% water, while the protein and carbohydrate

content varied between 4.7 and 6.6%, and 4.3 and 5.4%, respectively (Garrote *et al.*, 2001). The dry mass of the grains usually constitutes 10 - 16% of the whole grain. Approximately 30% of the dry mass is protein, while 25 - 50% are comprised of carbohydrates (Libudzisz & Piatkiewicz, 1990).

The microbes are embedded in and held together by kefiran, a matrix of resilient, fibrillar material composed primarily of insoluble polysaccharide material (La Rivière *et al.*, 1967; Marshall *et al.*, 1984; Marshall & Cole, 1985). It amounts to approximately 30 - 35% of the wet weight of the grains and is composed of equal amounts of galactose and glucose (Marshall, 1993; Özer & Özer, 2000).

Kefir grain microbial community

Kefir grains represent a natural symbiosis of different microbes (Duitschaever, 1989). Lactobacilli (homo- and hetero-fermentative, meso- or thermophilic) generally constitute approximately 65 - 80% of the total microbial population, while streptococci and lactococci comprise about 20% and lactose fermenting and non-lactose fermenting yeasts 5% of the total microbial population (Libudzisz & Piatkiewicz, 1990). The exact ratios of the microbes vary according to source and method of cultivation (Kurmann *et al.*, 1992; Özer & Özer, 2000).

Investigation of the grains by light or electron microscopy revealed that the microbes are not intermingled, but exist in a particular organized manner (Marshall *et al.*, 1984). Non-lactose-fermenting yeasts predominate in the core of the Kefir grains, while lactose-fermenting yeasts are found in the peripheral layers (Oberman & Libudzisz, 1998). The outer layer of the Kefir grains consists mainly of rod-shaped lactic acid bacteria. The intermediate areas contain a balance of yeasts and bacteria that changes evenly in relation to the distance from the core (Özer & Özer, 2000). Microbes reported to be present in the Kefir grains are listed in Tables 1 - 3.

Metabolism of microbes in Kefir grains

The unique flavour and aroma of traditional Kefir is the result of the complex symbiotic metabolic activity of the bacteria and yeast species present in Kefir grains (Beshkova *et al.*, 2003). A yeast-lactic fermentation occurs during the production of Kefir (Marshall, 1987; Litopoulou-Tzanetaki & Tzanetakis, 2000). In this type of fermentation the starter culture is primarily composed of mesophilic

Table 1. Lactobacilli present in Kefir grains.

Microorganism	Reference
Lactobacillus acidophilus	Varnam & Sutherland 1994; Litopoulou-Tzanetaki & Tzanetakis, 2000
Lb. brevis	Angulo *et al.*, 1993; Steinkraus, 1996; Motaghi *et al.*, 1997
Lb. casei	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
subsp. *alactosus*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
subsp. *pseudoplantarum*	Angulo *et al.*, 1993
subsp. *rhamnosus*	Angulo *et al.*, 1993; Marshall, 1993
Lb. casei	
subsp. *tolerans*	Angulo *et al.*, 1993
Lb. cellobiosus	Marshall, 1993; Özer & Özer, 2000
Lb. delbrueckii	
subsp. *bulgaricus*	Marshall, 1993; Özer & Özer, 2000
subsp. *lactis*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Lb. fermentum	Litopoulou-Tzanetaki & Tzanetakis, 2000; Özer & Özer, 2000
Lb. gasseri	Angulo *et al.*, 1993
Lb. helveticus	Koroleva, 1988a ; Lin *et al.*, 1999
subsp. *jugurti*	Libudzisz & Piatkiewicz, 1990; Özer & Özer, 2000
subsp. *lactis*	Marshall, 1993
Lb. kefir	Litopoulou-Tzanetaki & Tzanetakis, 2000; Garrote *et al.*, 2001
Lb. kefiranofaciens	Takizawa *et al.*, 1994; Litopoulou-Tzanetaki & Tzanetakis, 2000
Lb. kefirgranum	Takizawa, 1994; Garrote *et al.*, 1997; Takizawa, 1998
Lb. lactis	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
subsp. *lactis*	Özer & Özer, 2000
Lb. paracasei	
subsp. *paracasei*	Litopoulou-Tzanetaki & Tzanetakis, 2000
subsp. *tolerans*	Özer & Özer, 2000
Lb. parakefir	Takizawa, 1998; Özer & Özer, 2000; Garrote *et al.*, 2001
Lb. plantarum	Litopoulou-Tzanetaki & Tzanetakis, 2000; Garrote *et al.*, 2001
Lb. rhamnosus	Litopoulou-Tzanetaki & Tzanetakis, 2000
Lb. viridescens	Angulo *et al.*, 1993; Özer & Özer, 2000

Table 2. Lactococci, Streptococci, acetic acid bacteria and contaminants present in Kefir grains.

Microorganism	Reference
Lactococcus filant	Özer & Özer, 2000
Lac. lactis	Koroleva, 1988a
subsp. *cremoris*	Kurmann *et al.*, 1992; Varnam & Sutherland, 1994; Litopoulou-Tzanetaki & Tzanetakis, 2000; Özer & Özer, 2000
subsp. *lactis*	Motaghi *et al.*, 1997; Litopoulou-Tzanetaki & Tzanetakis, 2000; Özer & Özer, 2000; Garrote *et al.*, 2001
subsp. *lactis* var. *diacetylactis*	Libudzisz & Piatkiewicz, 1990; Litopoulou-Tzanetaki & Tzanetakis, 2000; Özer & Özer, 2000; Garrote *et al.*, 2001
Leuc. dextranicum	Özer & Özer, 2000
Leuc. kefir	Özer & Özer, 2000
Leuc. mesenteroides	
subsp. *cremoris*	Libudzisz & Piatkiewicz, 1990; Litopoulou-Tzanetaki & Tzanetakis, 2000
subsp. *dextranicum*	Libudzisz & Piatkiewicz, 1990; Litopoulou-Tzanetaki & Tzanetakis, 2000
subsp. *mesenteroides*	Litopoulou-Tzanetaki & Tzanetakis, 2000
Streptococcus durans	Libudzisz & Piatkiewicz, 1990
Str. salivarius subsp. *thermophilus*	Libudzisz & Piatkiewicz, 1990; Angulo *et all.*, 1993;Marshall, 1993
Acetic acid bacteria	
Acetobacter sp.	Angulo, 1993; Garrote *et al.*, 2001
A. aceti	Kurmann *et al.*, 1992; Marshall, 1993; Tamime *et al.*, 1999
A. rasens	Marshall, 1993; Tamime *et al.*, 1999
Contaminants	
Bacillus sp.	Angulo, 1993; Tamime *et al.*, 1999
Enterococcus sp.	Tamime *et al.*, 1999
Escherichia sp.	Angulo, 1993; Tamime *et al.*, 1999
Micrococcus sp.	Angulo, 1993; Tamime *et al.*, 1999
Pediococcus sp.	Angulo, 1993; Tamime *et al.*, 1999

Table 3. Yeasts and mycelial fungi present in Kefir grains.

Microorganism	Reference
Yeasts	
Brettanomyces anomalus	Garrote *et al.*, 1997; Lin *et al.*, 1999
Candida friedrichii	Angulo *et al.*, 1993; Özer & Özer, 2000
Can. kefir	Angulo, 1993; Steinkraus, 1996; Özer & Özer, 2000
Can. holmii	Marshall, 1993; Steinkraus, 1996; Özer & Özer, 2000
Can. pseudotropicalis	Libudzisz & Piatkiewicz, 1990; Özer & Özer, 2000
Can. tenius	Pintado *et al.*, 1996
Can. valida	Koroleva, 1988a; Özer & Özer, 2000
Debaryomyces hansenii	Loretan *et al.*, 2003
Kluyveromyces bulgaricus	Marshall, 1993; Özer & Özer, 2000
K. fragilis	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
K. lactis	Libudzisz & Piatkiewicz, 1990; Angulo *et* al., 1993; Marshall, 1993
K. marxianus subsp. *marxianus*	Kurmann *et al.*, 1992; Varnam & Sutherland, 1994; Özer & Özer, 2000
Mycotorula kefyr	Özer & Özer, 2000
M. lactis	Özer & Özer, 2000
Pichia sp.	Tamime *et al.*, 1999
P. fermentans	Lin *et al.*, 1999
Saccharomyces carlsbergensis	Libudzisz & Piatkiewicz, 1990
S. cerevisiae	Özer & Özer, 2000; Motaghi, 1997
S. dairensis	Özer & Özer, 2000
S. delbrueckii	Steinkraus, 1996; Angulo *et al.*, 1993; Libudzisz & Piatkiewicz, 1990
S. exiguus	Özer & Özer, 2000
S. florentinus	Libudzisz & Piatkiewicz, 1990
S. fragilis	Motaghi, 1997
S. globosus	Libudzisz & Piatkiewicz, 1990
S. globus	Özer & Özer, 2000
S. lactis	Motaghi, 1997
S. lipolytic	Garrote *et al.*, 1997
S. unisporus	Libudzisz & Piatkiewicz, 1990; Angulo, 1993; Özer & Özer, 2000
Torulaspora delbrueckii	Libudzisz & Piatkiewicz, 1990
Zygosaccharomyces florentinus	Özer & Özer, 2000
Z. rouxii	Loretan *et al.*, 2003
Mycelial fungi	
Geotrichum sp.	Garrote *et al.*, 1997; Tamime *et al.*, 1999
G. candidum	Tamime *et al.*, 1999

and thermophilic lactic acid bacteria strains combined with yeasts (Oberman & Libudzisz, 1998). Carbohydrates, organic acids and amino acids serve as substrates for the fermentation (Liu, 2003)

Lactose metabolism

Lactose is the primary energy source used by dairy lactic acid bacteria (Arihara & Luchansky, 1994). Lactose is a disaccharide composed of glucose and galactose and it is the only free-form sugar present in dairy milk (Johnson & Steele, 1997). During fermentation, the lactose in milk is converted to lactic acid leading to a decrease in the pH and a curdling of the milk. Up to 30% of the lactose is utilised and both D (-) lactic acid and L (+) lactic acid are produced (Rea *et al.*, 1996; Kuo & Lin, 1999). The ratio of L-lactic acid to D-lactic acid depends on the specific type of nicotinamide adenine dinucleotide (NAD^+) dependant lactate dehydrogenases (nLDH) present (Axelsson, 1998). In the Kefir beverage, the L (+) form predominates and the exact ratio depends on the microbial composition of the grains (Rea *et al.*, 1996; Özer & Özer, 2000). Lactococci produce only L-lactic acid, while *Lb. delbrueckii* subsp. *bulgaricus* and *Leuconostoc* spp. produce only D-lactic acid. Some lactobacilli possess both enzymes and produce D- and L-lactic acid (Johnson & Steele, 1997).

Lactose is utilised by homofermentative lactic acid bacteria, such as all lactococci and certain lactobacilli, producing lactate as an end-product, while heterofermentative lactic acid bacteria, including *Leuconostoc* spp. and certain lactobacilli, metabolise lactose and produce equal amounts of lactate, ethanol and carbon dioxide (Axelsson, 1998; Litopoulou-Tzanetaki & Tzanetakis, 2000). Lactose is also utilised by lactose fermenting yeasts through alcoholic fermentations (Litopoulou-Tzanetaki & Tzanetakis, 2000).

In Fig. 1 the metabolism of lactose by homofermentative microbes is depicted. The catabolism of lactose takes place inside the microbial cell and, therefore, the lactose has to be transported across the cell wall (Tamime & Robinson, 1985). It is transported across by means of the phosphoenolpyruvate-dependant: phosphotransferase system (PEP:PTS). Lactose is phophorylated during transport and once inside the cell, it is hydrolysed into D-glucose and D-galactose-6-phosphate by phospho-β-galactosidase (Arihara & Luchansky, 1995; Axelsson, 1998).

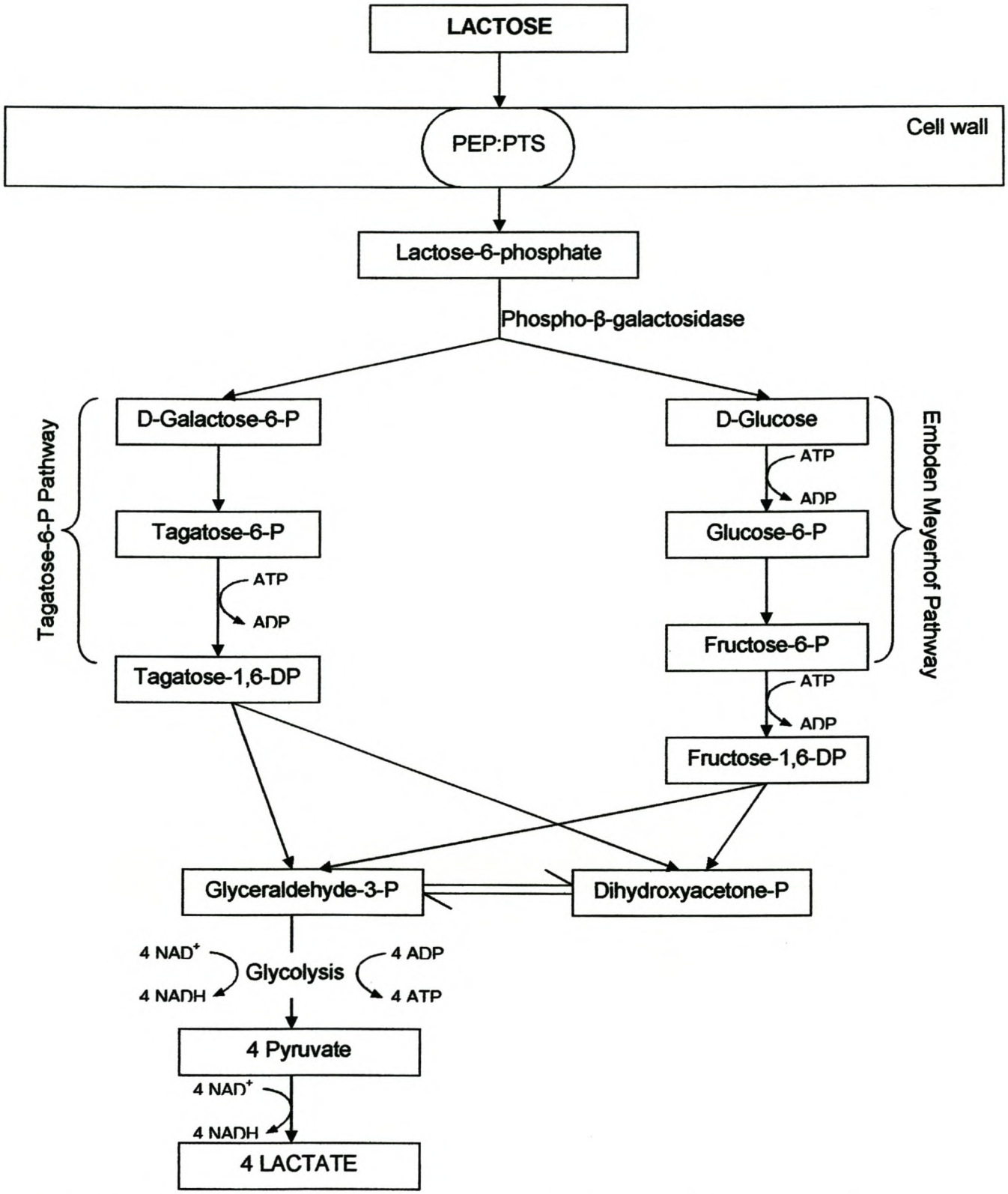

Figure 1. Pathways for lactose utilisation by homofermentative lactic acid bacteria (Arihara & Luchansky, 1995; Johnson & Steele, 1997; Axelsson, 1998).

Glucose is then metabolised to lactate following the Embden Meyerhof (glycolytic) pathway, which leads to the formation of fructose-1,6-diphosphate (FDP). The FDP is split into dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) by an FDP-aldolase. Both are then converted to pyruvate. Under normal conditions of excess sugar and limited access to oxygen, pyruvate is reduced to lactic acid by a NAD^+-dependant lactate dehydrogenase (nLDH) (Johnson & Steele, 1997).

Galactose-6-phosphate is metabolised to tagatose-6-phosphate via the tagatose-6-phosphate pathway (Tamime & Robinson, 1985; Arihara & Luchansky, 1995; Johnson & Steele, 1997; Litopoulou-Tzanetaki & Tzanetakis, 2000). It is then converted to triose phosphates, which is further metabolised by glycolysis to produce lactic acid (Arihara & Luchansky, 1995).

Lactose metabolism by heterofermentative microbes is as depicted in Fig. 2. Lactose is transported across the cell wall by a lactose carrier (permease) and is then hydrolysed to D-glucose and D-galactose by the enzyme β-galactosidase (Tamime & Robinson, 1985; Arihara & Luchansky, 1995; Axelsson, 1998; Farnworth & Mainville, 2003). The D-galactose is metabolised via the Leloir pathway, while the D-glucose is metabolised following the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (Axelsson, 1998; Litopoulou-Tzanetaki & Tzanetakis, 2000).

The Leloir pathway converts D-galactose to glucose-6-phosphate that is then further metabolised to lactic acid via glycolysis (Arihara & Luchansky, 1995). Certain bacteria, for example *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are unable to metabolise galactose. These bacteria release the galactose that can be assimilated and metabolised heterofermentatively by other bacteria (Johnson & Steele, 1997).

The 6-PG/PK pathway metabolises D-glucose by dehydrogenation and decarboxylation to form carbon dioxide and xylulose 5-phosphate, a pentose sugar. Xylulose 5-phosphate is split into GAP and acetyl phosphate by phosphoketolase.

GAP is metabolised via glycolysis to produce lactic acid, while acetyl phosphate is reduced to ethanol (Johnson & Steele, 1997; Axelsson, 1998). Certain *Leuconostoc* spp. convert acetyl phosphate into acetic acid during co-metabolism of lactose and citric acid (Johnson & Steele, 1997).

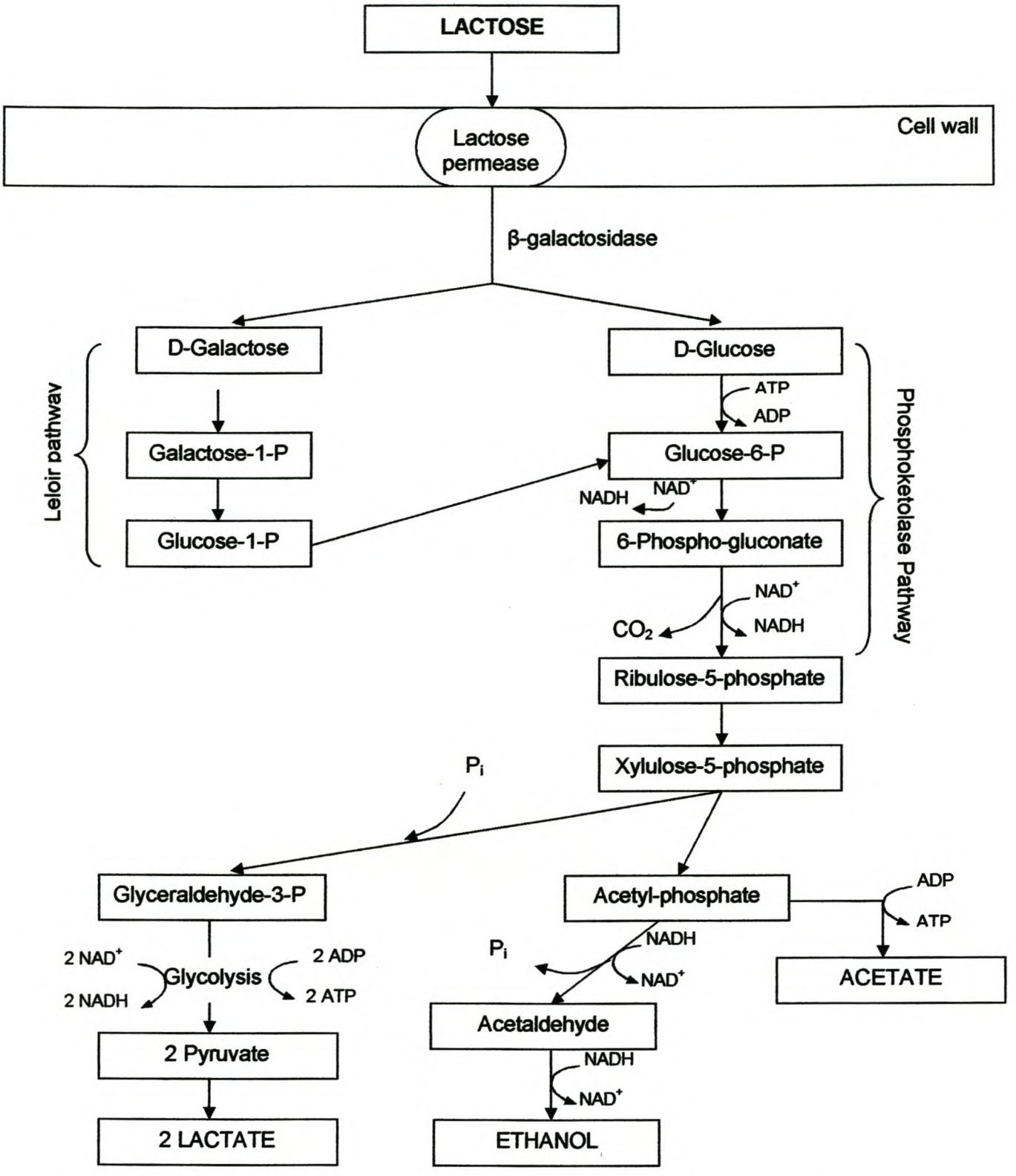

Figure 2. Pathways for lactose utilisation by heterofermentative lactic acid bacteria (Arihara & Luchansky, 1995; Johnson & Steele, 1997; Axelsson, 1998).

Citrate metabolism

Citrate is present as citric acid in milk at a concentration of 0.15 – 0.2%. It can be metabolised by various lactic acid bacteria, such as *Leuconostoc* spp. and *Lactococcus lactis* subsp. *lactis* and certain lactobacilli (Johnson & Steele, 1997; Axelsson, 1998). The catabolism of citrate usually results in the production of diacetyl, acetoin, butanediol and acetaldehyde, which have a distinct effect on the aroma of the fermented product (Hugenholtz, 1993).

Citrate is transported into the microbial cell by a citrate permease (Johnson & Steele, 1997). Once inside the cell, it is converted to oxaloacetate and acetic acid by citrate lyase (Hugenholtz, 1993). In *Lactococcus* and *Leuconostoc* spp. oxaleoacetate is decarboxylised by the enzyme oxaloacetate decarboxylase to form pyruvate (Driessen & Puhan, 1988; Johnson & Steele, 1997; Hugenholtz, 1993). Lactobacilli utilise citrate by at least two different metabolic pathways. *Lactobacillus* reduces oxaloacetate to succinate using part of the citric acid cycle (Cselovsky *et al.*, 1992). *Lactobacillus plantarum* can convert oxaloacetate to both succinate and pyruvate (Lindgren *et al.*, 1990; Kennes *et al.*, 1991).

Pyruvate metabolism

Although pyruvate is primarily reduced to lactic acid, lactic acid bacteria can also catabolise it to form various other compounds such as acetate, formate, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol and therefore contribute to the aroma of the fermented product (Liu, 2003). As is depicted in Fig. 3, lactic acid bacteria may change their utilisation of pyruvate to produce these compounds under certain environmental conditions (Axelsson, 1998).

The pathways leading to the formation of diacetyl and acetoin is common among lactic acid bacteria and plays an important role in the aroma of fermented milks. Pyruvate will only be catabolised by this pathway when there is a pyruvate surplus relative to the need for NAD^+ regeneration. This occurs when an alternative source, other than lactose, is present in the substrate, for example citric acid. Pyruvate can also be catabolised by this pathway when another compound apart from NAD^+ act as electron acceptor (Axelsson, 1998).

The pyruvate-formate lyase system only functions under anaerobic conditions. Pyruvate reacts with co-enzyme A (CoA) and forms formate and acetyl CoA. Acetyl CoA can function as an electron acceptor and be converted to

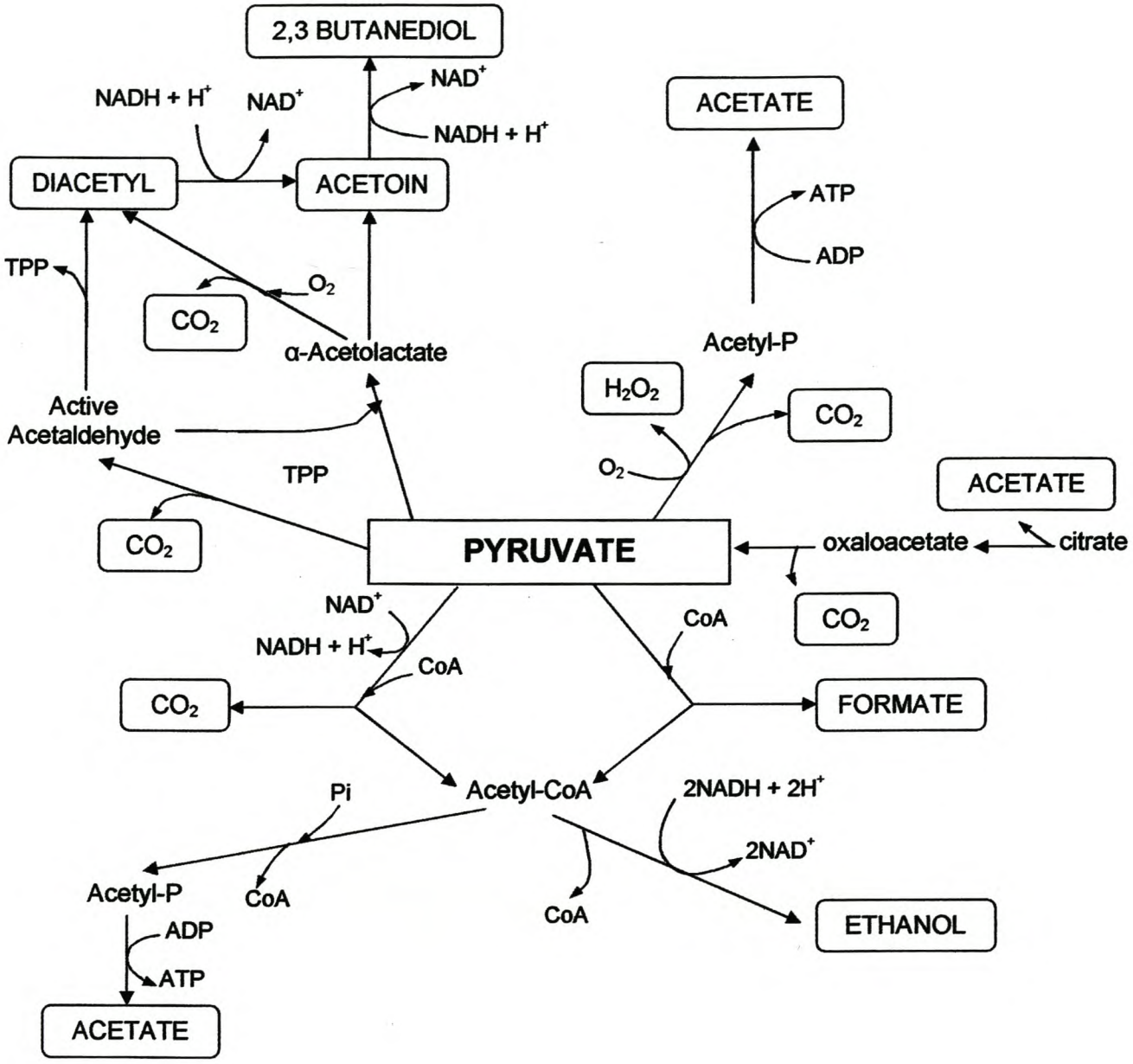

Figure 3. Alternative pathways for the utilisation of pyruvate. ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, co-enzyme A; NAD, nicotinamide adenine denucleotide; TPP, thiamin pyrophosphate (Driessen & Puhan, 1988; Johnson & Steele, 1997; Axelsson, 1998; Liu, 2003;).

ethanol while regenerating NAD^+. Alternatively, it can act as a precursor for substrate-level phosphorylation via acetyl phosphate, forming acetic acid and regenerating ATP (Johnson & Steele, 1997; Axelsson, 1998). In the presence of oxygen, a pyruvate dehydrogenase complex is active in lactococci and catalyses the conversion of pyruvate to acetyl-CoA and carbon dioxide (Axelsson, 1998).

Under aerobic conditions, certain microbes including *Lactobacillus plantarum* and *Lactococcus lactis* may convert pyruvate to acetyl phosphate and carbon dioxide with the formation of H_2O_2. Acetyl phosphate can then be metabolised further to form acetate (Axelsson, 1998).

Acetaldehyde production

Acetaldehyde is also one of the main volatile flavour components present in fermented milks (Johnson & Steele, 1997). Lactic acid bacteria are able to produce acetaldehyde through various pathways (Lees & Jargo, 1976). The cleavage of threonine into acetaldehyde and glycine by the enzyme threonine aldolase is considered as the most important pathway (Vandenbergh, 1993; Johnson & Steele, 1997). Microbes recorded to produce acetaldehyde include *Lactococcus lactis*, *Lac. cremoris*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Keenan *et al.*, 1966; Wilkins *et al.*, 1986). *Leuconostoc* spp. prevent excessive accumulation of acetaldehyde by metabolising it to ethanol (Johnson & Steele, 1997).

Proteolysis

Although most lactic acid bacteria are considered to be weak proteolytic, they do cause a significant degree of proteolysis during the fermentation of milk (Tamime & Robinson, 1985). Lactic acid bacteria are amino acid auxotrophs and require amino acids for growth. The amounts of free amino acids present in milk are not adequate and the microbes need proteolytic systems to utilize the peptides and proteins present in milk (Johnson & Steele, 1997).

Two classes of proteolytic enzymes are present in lactic acid bacteria, namely proteinases and peptidases (Thomas & Pritchard, 1987). Proteinases present in the cell wall of the microbe perform the first step of proteolysis by hydrolysing milk proteins. Once inside the cell, peptides are further degraded into free amino acids by peptidases (Arihara & Luchansky, 1995).

Proteolysis contributes to the flavour of fermented milks by the production of peptides, amino acids and derivatives thereof, including amines, acids, thiols, thioesters, as well as an increase in pH due to the formation of NH_3 and changes in the texture due to degradation of the protein matrix (Heller *et al.*, 2003).

Vitamin metabolism

Many lactic acid bacteria require B vitamins for growth, but several species are capable of synthesizing vitamins (Gurr, 1987). In Kefir, some vitamins are synthesized by both lactic acid bacteria and yeasts, while others are utilised by the Kefir microbes (Özer & Özer, 2000). Kneifel & Mayer (1991) determined that the thiamine (Vitamin B_1), pyridoxine (vitamin B_6) and folic acid concentration of milk increased with an average of 20% when fermented with grains to produce Kefir, while the orotic acid content decreased throughout the fermentation. Alm (1982) found that the Vitamin B_6, B_{12} and biotin content in Kefir decreased by 15% and the folic acid content increased by 40% after 1 day of storage. Vitamins B_3 and P (bioflavenoids) also accumulate in Kefir (Cataldo *et al.*, 1999; Özer & Özer, 2000; Anon., 2001).

Metabolism of the yeasts

Kefir grains contain both lactose fermenting and non-lactose fermenting yeasts that play and important role in the development of taste and aroma of the Kefir beverage (Koroleva, 1988a; Özer & Özer; 2000). Yeasts present in Kefir grains exert a favourable effect on the activity of the lactic acid bacteria by providing growth stimulants and metabolising some of the lactic acid (Koroleva, 1988a). The lactose fermenting yeasts in Kefir grains utilise pyruvate to form carbon dioxide and acetaldehyde. The acetaldehyde is then converted to ethanol (Ayres *et al.*, 1980).

Kefiran

Kefiran is synthesized along with the microbial growth during the incubation of the Kefir grains in milk (Steinkraus, 1996). It might be the most important metabolite produced by the microbes in the grains as it acts like a glue and keeps the grains intact (Özer & Özer, 2000). It has been found that encapsulated bacteria is present in propagatable grains and absent in non-propagatable grains.

This would suggest that encapsulated bacteria are responsible for the propagation of Kefir grains (Toba *et al.*, 1990).

A number of studies have been carried out to determine the microbe responsible for the production of kefiran (Marshall, 1993). It is thought to be the capsular material from certain lactobacilli present in the grains (Marshall *et al.*, 1984; Duitschaever, 1989; Yokoi *et al.*, 1990). La Rivière *et al.* (1967) found that kefiran is the capsular material of large rod-shaped bacteria that predominate in the grains and concluded that it was *Lactobacillus brevis* later regarded as *Lb. kefir* (Yokoi, *et al.*, 1990). A subsequent study could not replicate these results and speculated that *Lb. kefir* might not be the kefiran-producing microbe (Kandler & Kunath, 1983). Yokio, *et al.* (1990) found that the capsule-forming activity of *Lb. kefir* was lost after the first transfer to new growth medium when isolated. It has also been reported that ropy strains of *Lb. lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* and *Lb. casei* subsp. *casei* produce heteropolysaccharides when incubated in milk (Cerning *et al.*, 1992). *Lactobacillus kefiranofaciens* is also thought to be responsible for kefiran production (Marshall, 1993; Özer & Özer; 2000).

C. KEFIR PRODUCTION

History of Kefir production

In the northern Causasus region, raw bovine or caprine milk was used to produce Kefir in goat or sheep skin bags, clay pots or oak barrels by a continuous, uncontrolled fermentation (Kemp, 1984; Roginski, 1988; Libudzisz & Piatkiewicz, 1990; Tamime *et al.*, 1999). The bags were hung outside the house during winter and inside the house during summer (Duitschaever, 1989). It was hung near the door and anyone who entered or exited the house had to push the bag with their foot in order to mix the contents (Koroleva, 1988b). As the fermented milk was removed, it was replaced with fresh milk (Kemp, 1984). After continuous use of the same fermentation vessels, their walls became covered with an irregular layer of microbes resembling a film of Kefir grains (Roginski, 1988). Kefir produced in this way was characterised by a very high acidity and the carbon dioxide and ethanol content varied according to the length of time the milk was allowed to ferment (Koroleva, 1988b). It also had a distinct yeasty flavour (Roginski, 1988).

Commercially available starter cultures

In countries where Kefir is consumed, grains for household use can be purchased or obtained from other households that produce Kefir (Steinkraus, 1996). Commercially available Kefir grains are in the form of fresh grains, preserved in a sterile 0.9% (m/v) sodium chloride solution, or freeze-dried grains (Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Wszolek *et al.*, 2001). Fresh grains can be stored at 4°C for 8 - 10 days, while freeze-dried grains can be kept at room temperature for 12 - 18 months (Oberman & Libudzisz; 1998). Kefir tablets that are incubated in a glass of milk to produce a starter culture for subsequent fermentation, are less common (Libudzisz & Piatkiewicz, 1990).

Fresh grains are activated by adding them to heat-treated skim milk at a weight ratio of 1:10 (grains:milk). The mixture is then incubated at 20° ± 1°C for 24 h, where after the grains are sieved out. The fermented milk can then be used as a starter for subsequent fermentations (Libudzisz & Piatkiewicz, 1990).

Various methods exist for the activation of freeze-dried. One 1 g sachet of freeze-dried grains can be added to 3 litres of milk, which is then incubated at 20°C for 20 h. The resulting fermented milk acts as the starter culture for subsequent fermentations (Libudzisz & Piatkiewicz, 1990; Marshall, 1993). Alternatively, the grains can first be soaked for 2 - 3 h at 29 - 32°C in water followed by 2 - 3 h of soaking at the same temperature in water containing 1% (m/v) sodium bicarbonate. Grains that are swollen, elastic and translucent and tend to rise to the surface are then selected, rinsed and added to a bottle of sterilised milk. The inoculated milk is incubated at 18 - 21°C and the milk is replaced with fresh milk every 24 h. When gas bubbles are produced in the milk, the grains are ready for use (Whittier & Webb, 1950).

Pre-treatment of milk

Kefir can be made from whole, low fat or skim milk but often the fat content of the milk used is standardised (Marshall, 1993; Brewer, 1998; Wszolek *et al.*, 2001). Milk used for Kefir undergoes a heat treatment and homogenisation prior to fermentation. This treatment is more severe than normal pasteurisation and is designed to improve the product consistency by denaturing the whey proteins, resulting in a good coagulum and better mouth feel (Marshall, 1993; Varnam & Sutherland, 1994; Brewer, 1998). Different time-temperatures combinations used

for the heat treatment are depicted in Table 4. Denaturing of the whey proteins can also be achieved by an additional heat treatment. This typically consists of heating to 87°C, cooling to 77°C and maintaining constant temperature for 30 min before raising the temperature to 87°C. However, dairies prefer to use UHT milk to manufacture Kefir (Marshall, 1993).

Traditional production of Kefir

Kefir grains are added to heat treated milk and incubated at a specific temperature for a certain time or until the pH has decreased to a predetermined value. During this incubation period, the fermentation vessel may or may not be agitated to break the curd (Whittier & Webb, 1950; Steinkraus, 1996; Beshkova *et al.*, 2002). The grains are removed from the fermented milk, which can then either be cooled and consumed or it can be incubated at a lower temperature for a second fermentation (Koroleva, 1988b). During the second fermentation, an alcoholic fermentation takes place resulting in carbon dioxide production. This stage is known as the "ripening stage". After the ripening stage, the Kefir is ready for consumption (Roginski, 1988).

Procedures to produce Kefir vary greatly with regards to heat treatment of the milk, time and temperature of incubation, agitation of the fermentation vessel and ratio of grains to milk used. In Table 4 the parameters used in the production of Kefir are listed.

Industrial production of Kefir

The procedure of inoculating milk with Kefir grains and then collecting the grains from the fermented beverage is a very laborious practice when performed on a large scale (Kemp, 1984). For the production of Kefir on an industrial scale, a grainless starter culture is prepared as shown in Fig. 4 and used to inoculate milk (Roginski, 1988; Duitschaever, 1989).

Starter I or the "grain culture" is prepared by inoculating heat-treated milk with Kefir grains and incubating it as for the production of Kefir, followed by a ripening stage (Koroleva, 1988a). In some cases, the ripening step is omitted (Duitschaever, 1989). Starter I has the typical flavour and aroma of Kefir and it is recommended to use this starter as the inoculum to produce Kefir. In some circumstances a "bulk starter", Starter II is produced by fermenting heat-treated

Table 4. Variations of the operational parameters for the production of Kefir.

Heat treatment of milk		Inoculum (m/v)	Agitation	First fermentation		Second fermentation ("ripening stage")		Reference
Temperature (°C)	Time (min)			Temperature (°C)	Time (h)	Temperature (°C)	Time (h)	
121	10	nm	-	25	18	-	-	Loretan *et al.*, 2003
92	15	3%	3 - 5 stirrings	22	22	Slow cooling to 10°C	20	Beshkova *et al.*, 2002
85	25	5%	shaking: 90 rev/min	25	24	-	-	Assadi *et al.*, 2000
nm	nm	nm	-	20 - 22	10 - 12	Slow cooling to 8 - 10°C	10 - 12	Koroleva, 1988b
nm	nm	0.5 – 10%	-	20 - 23	12 - 24	10 - 15	24 - 72	Roginski, 1988
85	30	nm	-	22	over-night	-	-	Kosikowski, 1982
85	30	5 - 6%	-	18 - 25	24 - 48	24	8	Steinkraus, 1996
95	10 - 15	2 - 5%		18 - 22	24	-	-	Brewer, 1998
UHT		5%	gently stirred	23	24	-	-	Merin & Rosenthal, 1986
Pasteurised		2.5%	frequent shaking	14 - 16	8 - 10	14 - 16	24	Whittier & Webb, 1950

- = not included in process
nm = not mentioned in literature

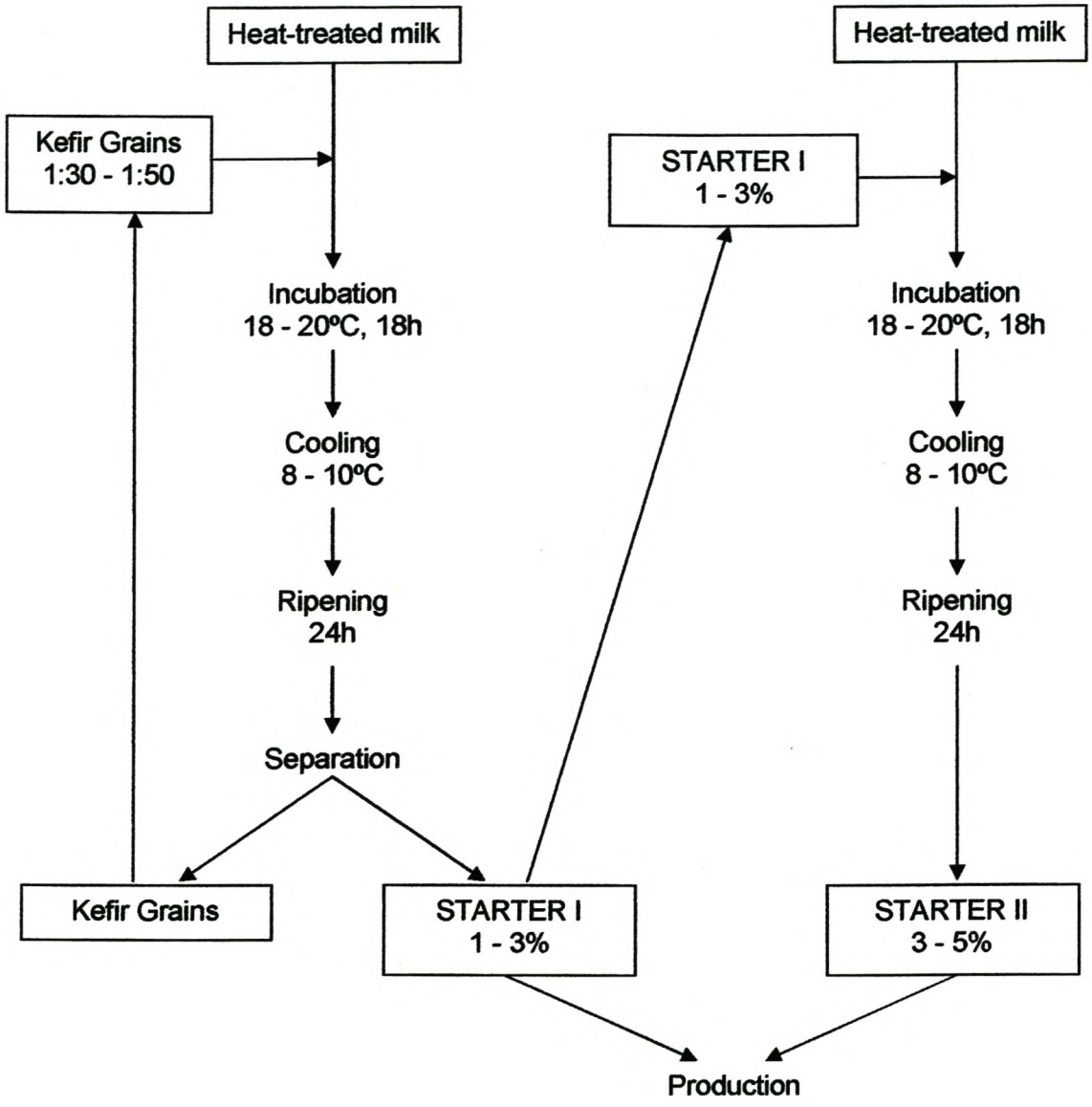

Figure 4. Preparation of the starters used for industrial Kefir production (Koroleva, 1988a).

milk with Starter I (Koroleva, 1988a). This is done because of a lack of adequate equipment for separating the Kefir grains from the fermented milk during the production of Starter I (Bavina, 1971). As is the case with traditional Kefir production, there are many variations for the production of Starters I and II with regards to the heat treatment of the milk, ratio of starter added, time and temperature of incubation, agitation of the fermenting milk and the presence of a ripening stage (Duitschaever, 1989; Kurmann *et al.*, 1992; Özer & Özer, 2000).

Factors affecting the Kefir microbes

The quality of the milk used for fermentation has an influence on the character of the final product. The milk should be free of antibiotics and disinfectant substances that could inhibit the microbial starter culture. Other factors include the age of the milk, citric and manganese content, heat treatment of the milk, homogenisation process and the fat content of the milk (Driessen & Puhan, 1988). Fermentation of milk with a low fat content can lead to a final product that lacks optimum body and mouth feel, which can be improved by adding 1 - 4% non-fat milk solids (skim milk powder) to the milk (Brewer, 1998).

Kefir produced with different grain:milk ratios differ in final pH, lactococci concentration, apparent viscosity and carbon dioxide content (Garrote *et al.*, 1998). Larger grain:milk ratios lead to a faster rate of acidification and shorter fermentation time, which coincides with a decrease in lactococci and yeast concentrations in the Kefir. Koroleva (1988a) claims that this is due to the shorter fermentation time, while Garrote *et al.* (1998) indicated that the lactococci decreased due to a high sensitivity to low pH conditions. When a smaller inoculum is used (for example $10g.l^{-1}$), the Kefir produced is less acidic, more viscous and the number of lactococci present in the Kefir is higher. It is thought that the higher concentration of lactococci is due to growth in the milk after the cells have been shed from the grains (Garrote *et al.*, 1998). The concentrations of thermophilic lactobacilli and acetic acid bacteria is not affected by the grain:milk ratio (Koroleva, 1998a).

The balance in which the microbes in the Kefir grains exist may be affected by agitation (Özer & Özer, 2000). Agitation during fermentation may result in an increase in the concentration of the homofermentative lactococci, streptococci and yeasts (Koroleva, 1988a; Özer & Özer, 2000). It has, however, no effect on the

lactobacilli, acetic acid bacteria and heterofermentative lactococci and streptococci concentrations. Agitation also does not have an effect on the quantity of the volatile fatty acids produced. It further prevents the growth of moulds on the surface of the substrate and causes an even distribution of microbial metabolites throughout the milk (Koroleva, 1988a).

Frequent washing of the grains with water leads to a decrease in the microbial numbers of the grains, a decreased activity and a longer fermentation time. The sensory attributes of the final product produced by washed grains do not represent that of Kefir. After washing, the balance of the microbes is only re-established after 3 - 5 days of cultivation (Koroleva, 1988a ; Özer &Özer, 2000).

The fermentation temperature plays an important role in the development of the characteristics of the final product. At high fermentation temperatures (25° - 27°C), the growth of homofermentative lactic acid bacteria (lactobacilli, lactococci and streptococci) is favoured. The activity of these microbes causes a rapid decrease in the pH and the required acidity is reached within 6 - 8 h (Koroleva 1988b). At the high acidity level homofermentative and heterofermentative lactic acid streptococci and lactococci are inhibited (Koroleva, 1988a). The heterofermentative organisms do not develop and results in Kefir with an atypical taste (Koroleva, 1988b). At fermentation temperatures of 20° - 22°C the required degree of acidity is reached within 10 - 12 h and if the product is then cooled to 8° - 10°C, the heterofermentative lactic acid bacteria and yeasts do not develop. If, instead, the product is cooled slowly during a 10 - 12 h period, the microbes will have sufficient time to develop the characteristic flavour and aroma of Kefir (Koroleva, 1988b).

D. COMMERCIALISATION

World-wide commercialisation of Kefir

Kefir is manufactured and marketed on a large scale in Russia, from where it originates (La Rivière, *et al.*, 1967). The knowledge and popularity of Kefir has spread and it is now produced commercially in various other countries including Poland, Germany, Sweden and Romania (Özer & Özer, 2000). It is currently available in the United States and is becoming increasingly popular in Japan (Saloff-Coste, 1996).

A problem encountered in the market place during the distribution of ready prepared Kefir, is the occurrence of secondary alcohol fermentation by the yeasts. This results not only in changes in the sensory characteristics, but ethanol and carbon dioxide continues to form leading to blowing of the containers and sequential leakage due the internal pressure (Kwak *et al.*, 1996). This problem can be overcome by producing Kefir with a lower yeast content, but such products lack the distinct effervescence and yeasty flavour associated with Kefir. Several closures that allow gas to escape have been designed for the containers in which the Kefir is sold (Marshall, 1993).

Apart from the packaging problems, difficulty is often experienced to produce Kefir of consistent quality (Kuo & Lin, 1999). This is because the specific balance and activity of the microbes are constantly changing and the Kefir produced from the same grains may vary from the one batch to the next (Kemp, 1984). Attempts have been made to produce Kefir from pure cultures that would allow better control over the microbes present. However, to date such attempts have failed (Marshall, 1985; Duitschaever, 1989; Özer & Özer, 2000). The quality of the Kefir can be improved by using two fermentation stages namely a lactic acid fermentation followed by an alcoholic fermentation (Mann, 1985). Due to the above-mentioned difficulties, it can be advantageous to commercialise the grains itself for household production of Kefir.

Fermented milk products in South Africa

The low-income South African consumer market has unique characteristics and requirements (Van Wyk *et al.*, 2002). Food products that are developed for a high-income market are not suitable for an economic environment where consumers hold a low purchase power (Bachmann, 1987). Requirements to which a product aimed at low-income consumers must comply include simple processing methods, the product has to be shelf stable under ambient temperature conditions until consumption, it must provide the essential nutritional elements and the product must also be complementary to the traditional local diet and the taste must be familiar or acceptable to the consumers (Bachmann, 1987).

The popularity of fermented milks has increased considerably in South Africa during the past thirty to forty years (Keller & Jordaan, 1990). Yoghurt,

drinking yoghurt, Maas and buttermilk are the most important cultured milk products, of which Maas is the principal traditional fermented milk (Keller & Jordaan, 1990; Joubert & De Lange, 1992; Hughson, 1995). Traditional Maas is made from raw (unpasteurised) milk. The milk is placed in containers such as calabashes or clay pots and allowed to ferment naturally. The same fermentation vessel is used repeatedly and with time a biofilm forms on the inner surfaces, containing a stable microbial population. The fermentation is performed chiefly by hetero- and homofermentative lactobacilli, streptococci, lactococci, leuconostocs and yeasts (Keller & Jordaan, 1990). Recent legislation stipulates that raw milk or raw cream may not be sold unless it is to be further processed (Anon., 1997). The production of Maas is not considered as further processing and low-income South African consumers cannot produce their own Maas due to the unavailability of raw milk (Viall, 1999). Commercial Maas produced from pasteurised milk, is a poor substitute for the traditional product, since it contains colourants, thickeners and preservatives and the taste is not comparable to that of traditional Maas (Berry, 1999; Van Wyk *et al.*, 2002).

A need exists for a fermented milk product that is low in cost, has a high nutritional value, is safe to consume and is similar in taste to traditional Maas. Kefir is a product that has the potential to fulfil these needs (Van Wyk, 2001). Kefir is easy to produce at home and does not require special equipment (Garrote *et* al., 1998; Van Wyk, 2001). It can be produced at room temperature and a variation in room temperature does not result in great variations in taste or the formation of strong off-flavours (Van Wyk, 2001). The taste of Kefir is comparable to that of traditional Maas and may be easily accepted by the target consumer (Van Wyk, 2001). Furthermore, Kefir is produced with re-usable grains and if handled correctly, they remain active for many years (Steinkraus, 1996). The consumer will purchase the grains once and the only expenses thereafter will be that of the milk (Van Wyk, 2002).

Mass cultivation of Kefir grains

During fermentation, Kefir grains increase in size and number and cultivation of the grains is performed by continuous cultivation in milk (Marshall & Cole, 1985). This can result in an increase in biomass of 5 - 7% per day (Libudzisz & Piatkiewicz, 1990). La Rivière *et al.* (1967) reported that 50 g Kefir

grains incubated in milk, adding up to a total volume of 500 ml, doubled in weight within 7 - 10 days when the milk was replaced 6 times a week. The results were similar irrespective of whether full cream, skim milk or whey was used.

Schoevers & Britz (2003) investigated the influence of different culturing conditions on the rate of Kefir grain biomass increase during mass-culturing. The variables examined were different incubation temperatures (18°, 22°, 25° and 30°C), milk enrichment with combinations of 2% (m/v) tryptose or 2% (m/v) yeast extract and 0.5% (m/v) urea, different milk volumes, different starter sizes and agitation of the fermentation vessel in a shake bath. It was found that the greatest weight increase occurred at 22°C, but fermentation at this temperature produced Kefir of low sensory quality. Subsequently 25°C was found to be the optimum incubation temperature. It was also found that the size of the initial starter inoculation had to be larger than 1% and that all the substrate had to be replaced daily. Enrichment of the substrate with a combination of urea and yeast extract resulted in a biomass increase seven fold more than obtained with conventional methods, while agitation led to a biomass increase six fold more than obtained without agitation.

Preservation of Kefir grains

Mass-culturing provides a means to obtain an adequate supply of grains for commercialisation. Mass-cultured grains must be preserved in an appropriate manner to allow distribution and long-term storage, while retaining their activity (Cilliers, 2001; Van Wyk, 2001). Kefir grains can be preserved by storage at 4°C for 8 - 10 days, storage at -20°C or drying of the grains (Kosikowski, 1982; Garrote *et* al., 1997; Özer & Özer, 2000). Kefir grains cannot be dehydrated by procedures involving elevated temperatures due to the heat-sensitivity of the microbes (Steinkraus, 1996). Instead, the grains are dehydrated by freeze-dried (Duitschaever, 1989; Marshall, 1993). Freeze-dried Kefir grains maintain their lactic acid activity and Kefir produced from these grains has bacterial counts similar to that of Kefir produced from fresh grains (Liu *et al.*, 1999). However, difficulties are encountered with the survival of the yeast component of the grains during freeze-drying (Duitschaever, 1989). Between 80 and 90% of the yeasts can be lost during freeze-drying (Marshall, 1993; Liu *et al.*, 1999). Such freeze-dried grains consist almost entirely of streptococci leading to a product that lacks the

effervescent characteristic and ethanol content of traditional Kefir (Glaeser, 1981; Marshall & Cole, 1985; Liu *et al.*, 1999). Freeze-dried Kefir grains are often standardized by the addition of yeast isolates and typically contains approximately 80% lactococci, 5% lactobacilli and 5% yeasts (Marshall, 1993; Liu *et al.*, 1999). Several factors affect the viability of the freeze-dried culture, namely the growth medium, the freezing rate, the drying temperature and the composition of the freezing medium. The viability is also affected by the storage conditions such as temperature, exposure to light and relative humidity after freeze-drying (Andersen *et al.*, 1999).

E. SENSORY CHARACTERISTICS AND EVALUATION OF KEFIR

The sensory properties of fermented milks should be mildly acidic and slightly prickly (Driessen & Puhan, 1988). Kefir has a sour flavour, also described as a clean, pleasant, acid taste or refreshing acidity that is contributed by various organic acids formed during fermentation, but especially lactic acid and acetic acid (Kemp, 1984; Duitschaever, 1989; Marshall, 1993). The prickly or effervescent sensation is due to the carbon dioxide formed by yeasts and heterofermentative organisms (Duitschaever, 1989; Keller & Jordaan; 1990). A buttery aroma should also be present due to the production of diacetyl (Marshall, 1993). The texture of Kefir is described as smooth, foamy and creamy (Kemp, 1984; Marshall, 1993). All of these properties contribute to an overall impression of a very refreshing beverage (Kemp, 1984).

Muir *et al.* (1999) compared the sensory profiles of buttermilk, yoghurt, traditional Kefir and modified Kefir with the use of descriptive sensory analysis techniques. Modified Kefir was made from a specific blend of microbes consisting of lactococci, lactobacilli and yeasts. Clear differences between the different products were found. The Kefir and buttermilk was found to be less viscous than the yoghurt and the perceived acidity was lower. The Kefir proved to be similar in many ways to buttermilk and both were perceived as being acid/sour and bitter. The serum separation for Kefir was more than that of the buttermilk. The modified Kefir differed from the traditional Kefir in that it was less acidic, had little serum separation and the flavour was creamier.

Wszolek *et al.* (2001) studied the properties of Kefir made in Scotland and Poland using bovine, caprine and ovine milk with different starters. It was found that the sensory attributes were influenced by the type of milk. Kefir beverages prepared from ovine and bovine milk were closer in character than those made from caprine milk and the flavour ranking was higher. Storage was found to influence the mouth feel characteristics ("serum separation", "chalky", "mouth-coating" and "slimy"). It was also concluded that the type of milk had a greater influence on the sensory profile than the starter cultures.

Van Wyk (2000) studied the sensory differences between Kefir, commercial Maas and Maas prepared in the laboratory with a commercial culture using a trained panel and a consumer panel. The results obtained from the trained panel indicated that the Kefir had a higher perceived acidity than both of the Maas samples, while "cowy" and "yeasty" tastes were more pronounced in commercial Maas than in Kefir and laboratory Maas. Kefir was less smooth than Maas and the most effervescent of the three products. From the results obtained by the consumer panel, it was concluded that Kefir is comparable to traditional Maas and is an appropriate substitute.

F. CONCLUSION

Kefir is a traditional fermented beverage produced by the fermentation of milk with Kefir grains. It is a very nutritious product and has been used in the past in the treatment of various illnesses and medical conditions. It is easy to prepare, does not require special equipment and can be produced at room temperature. The nutritional value and low-cost preparation of Kefir makes it ideal for the South African market. Furthermore, Kefir has the potential to function as a substitute for traditional Maas in the diet of low-income South African consumers and as the taste of Kefir is comparable to that of traditional Maas, it should be easily accepted by targeted consumers.

To commercialise the Kefir beverage, it will be necessary to effectively produce large quantities of active Kefir grains. Mass-cultured grains can then be preserved by freeze-drying in order to ease distribution and allow storage at room temperature until use. However, the production of the Kefir beverage from mass-cultured freeze-dried grains has not been studied. It is thus essential that the

freeze-dried mass-cultured Kefir grains be standardized and the production of the Kefir beverage using freeze-dried grains, optimised to produce Kefir with stable and excellent sensory characteristics.

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CHAPTER 3

OPTIMISATION OF THE PRODUCTION OF KEFIR BEVERAGE FROM MASS-CULTURED GRAINS

Abstract

Kefir is a refreshing, effervescent fermented milk beverage that has been consumed for centuries and is made by inoculating milk with Kefir grains. The aim of this study was to optimise the production of sensory acceptable Kefir from mass-cultured Kefir grains by varying the incubation times and temperatures, grain:milk ratios and heat-treatments of the milk used. It was found that defrosted mass-cultured grains had a liquid residue and mass loss studies indicated that storage at -18°C leads to a decrease in mass during defrosting and activation of the grains. Kefir with the best sensory results of nine production methods tested, was obtained after activation of the grains at 22°C for two successive 24 h periods, followed by Kefir production at 22°C for 18 h and a ripening stage at 18°C for 6 h. The milk was replaced after each incubation period and the fermentation vessel was agitated at the start of the incubation and after 12 h. Descriptive sensory analyses were performed on Kefir beverages prepared with different grain:milk ratios and heat-treated milks. Kefir prepared with 108 g grains.l^{-1} was less smooth, had a thinner consistency and was more yeasty, effervescent and sour than Kefir beverages prepared with 36 and 72 g grains.l^{-1}. The beverages prepared with 36 and 72 g grains.l^{-1} were very similar and had a better overall acceptability. Kefir produced from pasteurised milk was significantly less smooth, creamy and sweet, but more sour than Kefir produced from double pasteurised and ultra high temperature milk (UHT). There were no significant differences between Kefir produced from double pasteurised and UHT milks, although they had superior mouth feel qualities compared to the beverage produced from pasteurised milk.

Introduction

Kefir is a traditional fermented milk beverage originating from the Caucasian mountains in Russia (Kemp, 1984; Garrote *et al.*, 2000). It has a characteristic

taste and aroma, and is described as being sour, yeasty and refreshing (Duitschaever, 1989; Kurmann *et al.*, 1992; Marshall, 1993). It is slightly alcoholic, with a creamy consistency and a distinct effervescence (Kurmann *et al.*, 1992; Oberman & Libudzisz, 1998). Kefir generally has a pH of 3 - 4 and a lactic acid content of 0.68 - 1.5% (Duitschaever *et al.*, 1987; Steinkraus, 1996).

Kefir is not produced from a starter culture that is evenly distributed throughout the milk, but from re-usable grains (Marshall, 1993). These grains consist of a complex mixture of microbes embedded in kefiran, a polysaccharide matrix (La Rivière *et al.*, 1967; Saloff-Coste, 1996). The microbes exist in a symbiotic relationship and consist mainly of lactobacilli, lactococci, streptococci and yeasts (Libudzisz & Piatkiewicz, 1990; Saloff-Coste, 1996).

Household Kefir is produced by adding the grains to pasteurised milk, followed by incubation at temperatures ranging from 18° - 25°C (Steinkraus, 1996). During this stage, the milk can be agitated to increase the concentration of homofermentative lactococci, streptococci and yeasts and to break the curd (Whittier & Webb, 1950; Beshkova; 2002). After an incubation period of 12 - 48 h, the grains are removed and the fermented milk beverage can either be consumed or incubated for a ripening period at a lower temperature, when an alcoholic fermentation takes place (Roginski, 1988; Steinkraus, 1996).

The characteristics of the Kefir beverage may be influenced by numerous factors. The age, heat treatment and fat content of the milk, as well as the type of milk used effects the properties of the fermented product (Driessen & Puhan, 1988; Wszolek *et al.*, 2001). The specific grain:milk ratio, incubation times and temperatures, origin of the grains and agitation of the fermentation vessel also have an influence on the quality of the Kefir. (Koroleva, 1988b; Marshall, 1993; Garrote *et al.*, 1998; Özer & Özer, 2000).

The influence of different culturing conditions on the Kefir grain biomass increase was studied by Schoevers & Britz (2003) and a successful method for the rapid production of large quantities of mass-cultured Kefir grains was developed. However, mass-culturing has an effect on the microbial composition of the grains and will therefore influence the sensory characteristics of Kefir produced from these grains (Witthuhn *et al.*, 2004). The production of Kefir from these grains and the characteristics of such a beverage have not been investigated.

The aim of this study was to optimise Kefir beverage production using mass-cultured Kefir grains by examining the effect of different fermentation time and temperature combinations. Descriptive sensory analyses were performed on Kefir produced with different grain:milk ratios and heat-treated milks to characterise these beverages.

Materials and methods

Mass-culture of Kefir grains and mass loss during storage

Frozen Kefir grains were obtained from the University of Stellenbosch Food Science Culture Collection and mass-cultured according to the method developed by Schoevers & Britz (2003). To prepare the mass-culturing medium, pasteurised full cream milk was obtained from local supermarkets to which 20 $g.l^{-1}$ yeast extract (Merck) and 5 $g.l^{-1}$ urea (Merck) were added. The mixture was heat-treated in a temperature-controlled waterbath at 80° ± 2°C for 90 min, where after the medium was cooled to room temperature before use.

Sterile 1 l containers were filled with 400 ml of the milk medium and 40 g of defrosted Kefir grains were added. The containers were constantly agitated in a waterbath at 130 rpm at 25° ± 1°C. The grains were recovered from the medium after every 24 h of incubation by passing it though a sterilised household sieve. The grains were then inoculated into 400 ml fresh milk medium and the produced grains were removed every 7 d and stored at -18°C for no longer than 3 months.

When Kefir grains were subjected to frozen storage, a decrease in the mass of the grains was detected and the effect of storage time on the mass decrease of mass-cultured grains was investigated. Twenty grams of mass-cultured grains were stored at -18°C for 1, 2 and 3 weeks. The duplicate grain samples were defrosted, excess moisture was removed by sieving, the grains were weighed and the mass decrease was calculated.

Production of Kefir

Pasteurised milk obtained from a local supermarket was heat-treated (double pasteurised) in a temperature-controlled waterbath at 85° ± 2°C for 20 min.

The milk (250 ml) was transferred to 500 ml sterile containers and allowed to cool to room temperature.

Defrosted mass-cultured grains were activated in the cooled milk at a concentration of 72 g grains.l^{-1}. The mixture was then incubated for a number of incubation periods at nine different time and temperature combinations (Table 1). After every incubation period, the grains were recovered from the medium by sieving, followed by inoculation into 250 ml of fresh double pasteurised milk. The activated Kefir grains were used to produce Kefir by inoculating into 250 ml double pasteurised milk, followed by incubation. After the initial incubation period, the grains were removed and the fermented milk was incubated for a maturation period.

Method 9 (Table 1) differed from the other methods in that the fermentation vessel was swirled 5 times directly after inoculation and again after 12 h of incubation. This was done to increase the numbers of the homofermentative lactococci, streptococci and yeasts, which leads to a shorter activation period and a more effervescent final product (Koroleva, 1988a; Özer & Özer, 2000).

The pH of the final beverages were measured with a Mettler Toledo 320 pH meter and the TA was measured in duplicate by the titration of a 9 ml sample with 0.1 N NaOH to the faint pink phenolphtalein endpoint (Dixon, 1973). The sensory characteristics of the nine final beverages were evaluated individually by an experienced sensory panel consisting of three members with regards to flavour, acidity, effervescence and consistency.

Mass changes of mass-cultured Kefir grains during activation and Kefir production

In the preliminary tests, it was found that the mass of the Kefir grains decreases during activation and production of Kefir. Therefore, the changes in the mass of the grains were determined by measuring the mass before and after every incubation period. Kefir grains were activated and Kefir was produced following procedures for Methods 1, 2 and 3 with an inoculum of 72 g grains.l^{-1}, Method 4 with inoculums of 72 g.l^{-1} and 144 g.l^{-1} of mass-cultured grains and with previously frozen and fresh mass-grains using Method 9 with an inoculum of 36 g grains.l^{-1}. The experiments were conducted in duplicate and the final mass of the grains was expressed as a percentage of the original mass of the grains (m/m).

Table 1. Different incubation time and temperature combinations for the production of Kefir and the sensory properties, final pH and TA of Kefir produced with an inoculum of 72 g grains.l^{-1}.

Method	Activation	Incubation	Maturation	Sensory characteristics				Final pH	Final TA (%)
				Flavour	Acidity	Effervescence	Consistency		
1	4 x 18h @ 22ºC	16h @ 22ºC	6h @ 18ºC	-	(+)	-	-	4.21	0.87
2	4 x 18h @ 22°C 1 x 18h @ 25°C	18h @ 22ºC	6h @ 18ºC	-	-	-	-	4.33	0.70
3	2 x 18h @ 22°C 1 x 16h @ 25°C 1 x 18h @ 22°C 1 x 16h @ 18°C	18h @ 22°C	6h @ 18ºC	-	-	-	-	4.36	0.66
4	4 x 18h @ 22°C	18h @ 22°C	6h @ 18ºC	(+)	(+)	-	(+)	4.25	0.76
5	2 x 24h @ 22ºC 1 x 18h @ 22ºC 1 x 24h @ 22ºC	18h @ 22°C	6h @ 18ºC	-	-	-	+	4.22	0.86
6	2 x 24h @ 22ºC 1 x 18h @ 22ºC 1 x 24h @ 22ºC	18h @ 22°C	10h @ 18ºC	(+)	(+)	(+)	(+)	4.17	0.75
7	4 x 18h @ 22ºC 1 x 24h @ 22ºC	18h @ 22°C	6h @ 18ºC	-	-	-	+	4.23	0.75
8	4 x 18h @ 22ºC 1 x 24h @ 22ºC	18h @ 22°C	10h @ 18ºC	-	-	-	+	4.19	0.68
9	2 x 24 h @ 22ºC with agitation	18h @ 22°C	6h @ 18ºC	(+)	+	(+)	+	4.33	0.70

– = not present/ not enough
(+) = present, but not optimal
\+ = satisfactorily present

Changes in pH and TA during activation and production of Kefir

The final pH and TA values of the Kefir beverage will have an influence on the sensory properties and is also an indication of the level of activity of the microbes in the Kefir grains. The changes in pH and TA of the fermented milk were determined in duplicate during the activation of the mass-cultured Kefir grains and the production of Kefir using different methods, inoculum sizes and heat-treated milks in the following studies.

Mass-cultured Kefir grains were activated and Kefir was produced using Methods 2, 3, 4 and 9. An inoculum size of 72 g grains.l^{-1} was used.

Eighteen, 27, 36 and 54 g of grains were inoculated in 500 ml of double pasteurised milk (36, 54, 72 and 108 g grains.l^{-1}) in duplicate. The grains were activated and used to produce Kefir according to Method 9. The mass of the grains was kept constant by supplementing with grains of the same degree of activity or by the removal of excess grains. Kefir was produced in duplicate with Method 9 using an inoculum of 36 g grains.l^{-1} not kept constant allowing the mass to fluctuate naturally. Fresh mass-cultured grains that were activated and used to produce Kefir following Method 9 with an inoculum size of 36 g grains.l^{-1} to serve as a control.

Kefir was produced in duplicate using Method 9 by inoculating 18 g of grains in 500 ml pasteurised and UHT milk (36 g grains.l^{-1}) in 1 l sterile containers. The mass of the grains was kept constant.

Sensory evaluations of Kefir

Sensory evaluation method - The Kefir samples were evaluated using quantitative descriptive analysis (QDA) (Stone *et al.*, 1974). Eight panellists were recruited from staff and post-graduate students from the Department of Food Science at the Stellenbosch University and trained. The development of the experimental vocabulary took place during the initial training sessions by means of modifying the vocabulary used in similar sensory analyses of Kefir and Maas (Human, 1998; Muir *et al.*, 1999; Wszolek *et al.*, 2001), as well as suggestions from the panel members. The descriptors, classified into four groups namely appearance, taste, texture and acceptability, are listed in Table 2. Perceived visual smoothness was designated "Smoothness 1", while perceived oral smoothness was designated "Smoothness 2".

Table 2. Descriptors used for the sensory evaluation of Kefir.

Descriptor	Scale (0 →100)	Definition
Appearance		
Smoothness 1	gritty → extremely smooth	Grittiness on the back of a metal spoon dipped in the sample
Thickness	runny → drinking yoghurt	Degree of thickness measured by spooning the sample
Flavour		
Boiled milk	none → extreme	Flavour of boiled milk
Yeasty	none → extreme	Yeasty flavour
Sweetness	none → extreme	Sweet taste
Sourness	none → extreme	Sour taste
Texture		
Prickly/Effervescence	none → extreme	Prickly feeling on the tongue
Creamy mouth feel	watery → extremely creamy	Degree of creamy mouth coating
Smoothness 2	gritty → extremely smooth	Perceived smoothness of the sample in the mouth
Chalky mouth feel	none → extreme	Floury-dry after-taste
Acceptability		
Overall acceptability	unacceptable → extremely acceptable	Perceived liking of the sample

The sensory evaluation and sample placement forms used are given in Appendix A at the end of the chapter. The intensity ratings were scored on a 10 cm unipolar unstructured line scale, with verbal anchors at either side. During evaluation, the panellists were asked to place a vertical line across the horizontal line at a point that best quantified their perception of intensity. The panellist responses were converted to a scale of 0 to 100. Sensory evaluation was carried out under fluorescent lighting conditions in an adequately ventilated area. Approximately 50 ml of each sample was presented in 125 ml white styrofoam cups covered with foil and stainless steel teaspoons were provided to assist in the evaluation. Each sample was assigned a random three-digit code and the samples were presented in random order regarding replicate and panellist. Panellists were asked to refresh their palates between every sample with distilled water and table water biscuits.

Sensory Study I - Comparison of sensory characteristics of Kefir prepared with different grain:milk ratios

Mass-cultured grains were defrosted, activated and used to make three different Kefir beverages using Method 9 with inoculums of 36, 72 and 108 g of grains.l^{-1} of milk, respectively. Activation and production of the Kefir was performed in 1 l sterile containers containing 500 ml double pasteurised milk.

Sensory Study II - Comparison of sensory characteristics of Kefir prepared from different heat-treated milks.

Mass-cultured grains were defrosted, activated and three different Kefir beverages were prepared using Method 9 with an inoculum of 36 g.l^{-1} Kefir grains using pasteurised (P), double pasteurised (DP) and UHT milk. Activation and production of Kefir was performed in 1 l sterile containers containing 500 ml of milk.

The Kefir beverages prepared for Sensory Studies I and II were refrigerated (4°C) overnight (± 18 h) and presented to a sensory panel. In the case of Study I, it was presented to the panel of eight trained members for three consecutive tasting sessions, and during Study II, the samples were presented to a panel of seven members in four consecutive tasting sessions. Half hour breaks were taken

between each individual tasting session. The pH and TA of the final beverages were measured.

Statistical analysis

One-way analysis of variance (ANOVA) was done for each sensory study using Statistica™ 6 (version 6.1.409) for Windows™. The sources of variance were different inoculum sizes in Sensory Study 1 and different heat-treated milks in Sensory Study 2. Mean values were considered significantly different at $p \leq 0.05$. If the samples differed significantly, an *ad hoc* test, Bonferroni, was performed to determine which samples differed. In the case of the residual values not conforming to a norm curve, a non-parametric test, Kruskal-Wallis, was performed. Radar plots were drawn using Excel™ 2000 (version 9.0.2720) for Windows™.

Results and discussion

The large volume of data generated in this study is presented at the end of this chapter as Appendix B.

Mass loss of Kefir grains during freezing

The mass loss of Kefir grains during freezing was determined, as it was found that defrosted grains released a liquid residue as is shown in Fig. 1. The percentages mass loss (m/m) after 1, 2 and 3 weeks of frozen storage were 10.97, 20.75 and 21.34%, respectively. It is thus not recommended to freeze Kefir grains or to freeze them for a short period of time as the mass of the grains decreases when the grains are defrosted.

Production of Kefir

Kefir produced using Method 1 (Table 1) resulted in a beverage that had pleasant sensory properties, but a too runny consistency. The Kefir did not have enough effervescence and did not contain enough flavour components. The perceived acidity of the Kefir was adequate. Kefir beverages produced by Methods 2 and 3 both lacked flavour components, effervescence, sourness and had a runny consistency.

Figure 1. Defrosted mass-cultured Kefir grains.

Kefir produced by Method 4 resulted in a beverage that had better sensory properties than the Kefir produced using Methods 1 - 3. Although flavour substances could be detected, it was still not satisfactorily. The product had a pleasant sourness, but was not effervescent and thick enough.

Methods 5 and 7 produced beverages that had thick consistencies, no sourness and did not have sufficient flavour components or effervescence. Method 6 produced a Kefir that was thick and had some flavour and effervescence, while Method 8 resulted in a Kefir that had no flavour, effervescence and sourness, with a thick consistency.

Producing Kefir using Methods 1 - 8 requires replacing the milk at night once or twice during the incubation and production of Kefir. Since this would be burdensome to the consumer, Method 9 was developed that differed from the other methods in that the fermentation vessel was agitated. This was done in an effort to shorten the activation time and increase the effervescence of the final product (Koroleva, 1988a; Özer & Özer, 2000).

Kefir produced by Method 9 had a high perceived acidity, a thick consistency and the desirable flavour and effervescence could be detected. Method 9 was identified as a method that produces Kefir with satisfactorily sensory characteristics and it was used in further sensory experimental studies.

The final pH values of the nine methods ranged between 4.17 and 4.34 and the titratable acidity between 0.66 and 0.86% (Table 1). According to Marshall (1993), the pH of traditional Kefir should be 4.4 or higher, but the modern consumer prefers a Kefir with a lower pH. Therefore, the pH values for the nine methods were all within the desired pH range. The titratable acidity of commercially available Kefir is approximately 1% (Marshall, 1993), which is higher than the titratable acidity of the Kefir produced by Methods 1 – 9. In this study, the grains were only activated until they were active enough to produce Kefir with acceptable sensory properties. It has been reported that during continuous production of Kefir from the active grains, the TA increased as the activity of the grains grains increases (Schoevers & Britz, 2003).

Mass changes of mass-cultured Kefir grains during activation and Kefir production

The measured mass values are as depicted in Tables B1 to B3 of Appendix B. The changes in the grain mass is more likely due to the length of frozen

storage of the grains than the specific production method. Grains used in the production of Kefir with Methods 1 - 3 were frozen for 3 months prior to activation. The mass of the grains decreased during activation and production of Kefir with Methods 1, 2 and 3 (69, 46 and 66% of the original mass, respectively). The grains used in the production of Kefir using Method 4 were frozen for 1 week and increased slightly (104 and 114% of the original mass). It can be deduced that frozen storage led to a loss in the mass of the grains during activation.

To prove that freezing of the mass-cultured grains lead to a decrease in mass during the activation, grains were activated and Kefir produced with grains that were frozen for three months and fresh mass-cultured grains. Method 9 and an inoculum size of 36 g grains.l^{-1} were used and the results are presented in Table B3. The mass of the previously frozen grains decreased (37 and 42% of the original mass), while the mass of the freshly mass-cultured grains increased (184 and 168% of the original mass). This confirms that freezing of mass-cultured grains prior to activation and production of Kefir leads to a decrease in grain mass.

Changes in pH and TA during activation and production of Kefir

The data in Fig. 2 shows the changes in pH and TA for the activation and production of Kefir by Methods 2, 3, 4 and 9 with an inoculum size of 72 g grains.l^{-1}. An acceptable pH and TA level was reached for all the methods after 70 h, although it was reached within a shorter activation period with Methods 2 and 3.

The changes in pH and TA of the milk during the activation and production of Kefir using Method 9 with inoculum sizes of 36, 54, 72 and 108 g grains.l^{-1} are depicted in Fig. 3. Although the pH and TA values varied greatly after 24 h, the final values did not differ considerably, indicating that the inoculum size did not play a significant role in the final pH and TA values. The changes in pH and TA were very similar when the inoculum size was kept constant and allowed to fluctuate naturally. The mass changes of the grains used to make Kefir without keeping the inoculum size constant are depicted in Table B3. It appears that the decrease in the mass of this sample did not retard the acidification. This confirms that inoculum size does not play a role in the final pH and TA of the Kefir beverage.

The changes in pH and TA during the activation of grains and production of fresh mass-cultured grains (control) versus grains that had been frozen for 3

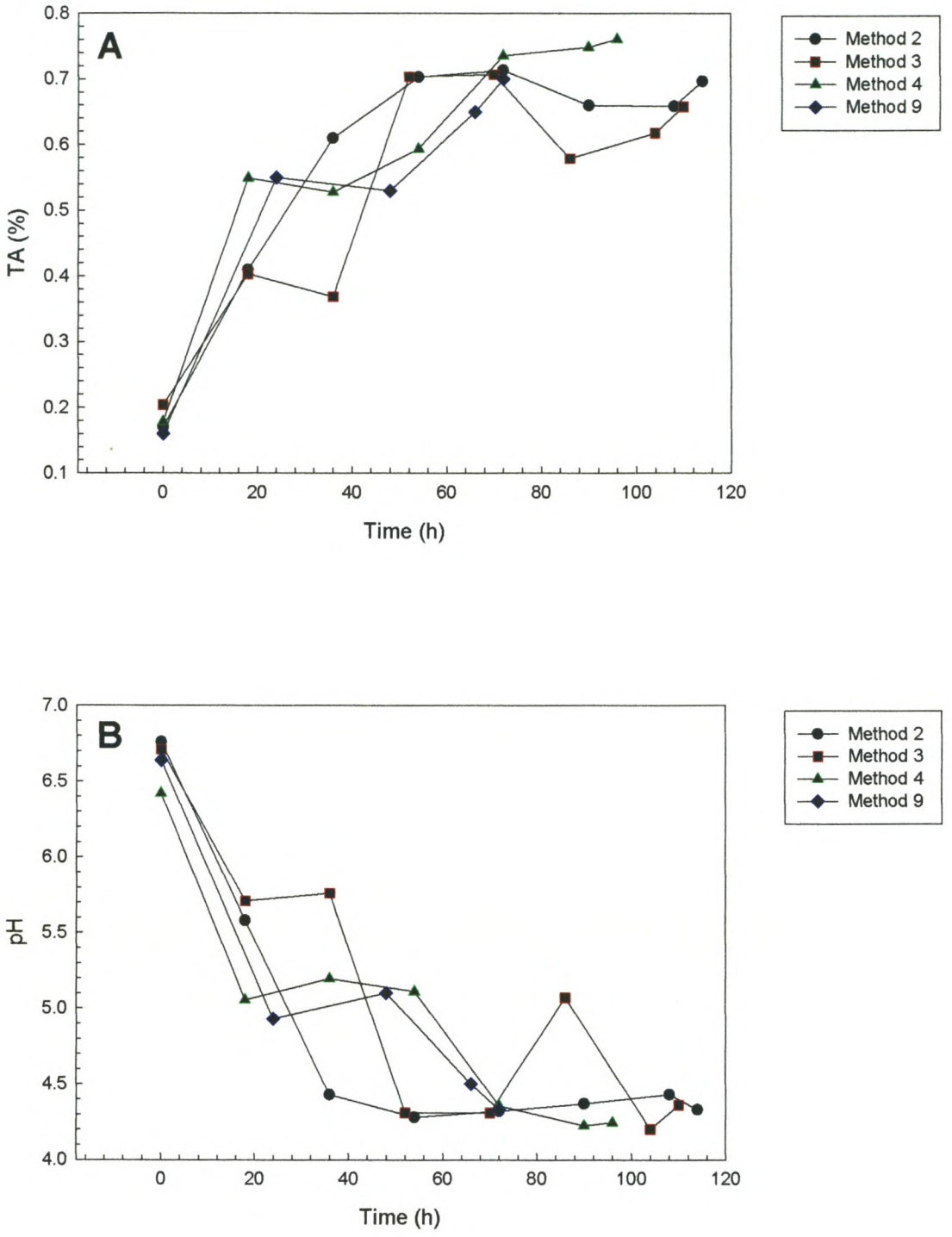

Figure 2. Changes in the pH (**A**) and TA (**B**) during the activation of mass-cultured Kefir grains and production of Kefir in DP milk using Methods 2, 3, 4 and 9 with an inoculum of 72 g grains.l^{-1}.

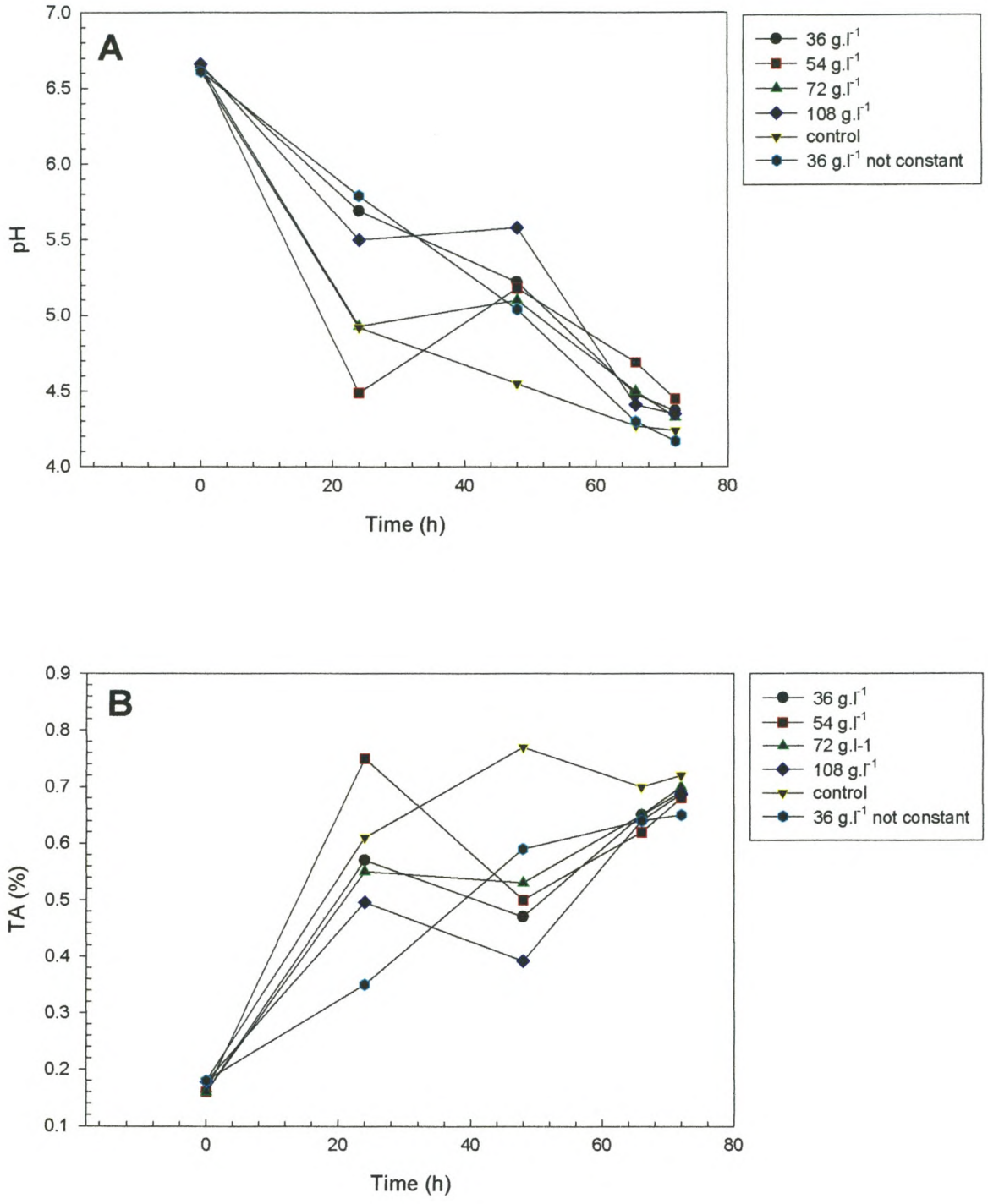

Figure 3. Changes in the pH (**A**) and TA (**B**) during the activation of mass-cultured Kefir grains and the production of Kefir in DP milk using Method 9 with different inoculum sizes (control = 36 $g.l^{-1}$ of fresh mass-cultured grains).

months showed a variation during the initial activation period (Fig. 3). Although the variation is less in the final Kefir beverages, it is clear that the Kefir produced from grains that have not been frozen, had a higher acidity than Kefir produced from grains that have been frozen.

The effect of the heat-treatment of the milk, (pasteurisation (P), double pasteurisation (DP) and ultra high temperature treatment (UHT)), on the changes in pH and TA during the activation of Kefir grains and the production of Kefir was investigated and the results are illustrated in Fig. 4. Method 9 was used with an inoculum size of 36 g grains.l^{-1}. Differences were observed in the final pH values of Kefir made from P, DP and UHT milks, namely 4.64, 4.15 and 4.97, respectively. The TA, namely 0.70, 0.66 and 0.65%, for P, DP and UHT respectively, did not differ considerably. The consumer prefers a Kefir with a pH lower than 4.4 (Marshall, 1993) and the DP milk was identified as the preferable heat-treated milk to be used for the production of Kepi.

Sensory evaluation of Kefir produced with different grain:milk ratios and different heat-treated milks

Sensory Study I - Comparison of sensory characteristics of Kefir prepared with different grain:milk ratios

This study was undertaken to determine the effect of the grain:milk ratio in the production of Kefir on the sensory characteristics of the final product. The means and *p*-values of the sensory attributes evaluated are shown in Table 3 and the radar plot in Fig. 5. The summarised results of the analyses of variances are depicted in Table B4. The three Kefir beverages differed significantly with regards to all characteristics, except smoothness 1 and chalky mouth feel (Table 3). Kefir prepared with 108 g grains.l^{-1} differed the most and it was perceived as being less thick. This correlates with the results of Garrote *et al.* (1998) who found that Kefir prepared with 100 g grains.l^{-1} had a lower viscosity than Kefir prepared from 20 and 50 g grains.l^{-1}. Kefir prepared with 108 g grains.l^{-1} had less oral smoothness and was significantly more yeasty and sour than the other two beverages (Fig. 5). This could be subscribed to the higher concentration of microbes present, producing more acid and yeasty-like aroma. This also explains why the sweetness and boiled milk taste decreased as the size of the inoculum increased. The yeasts

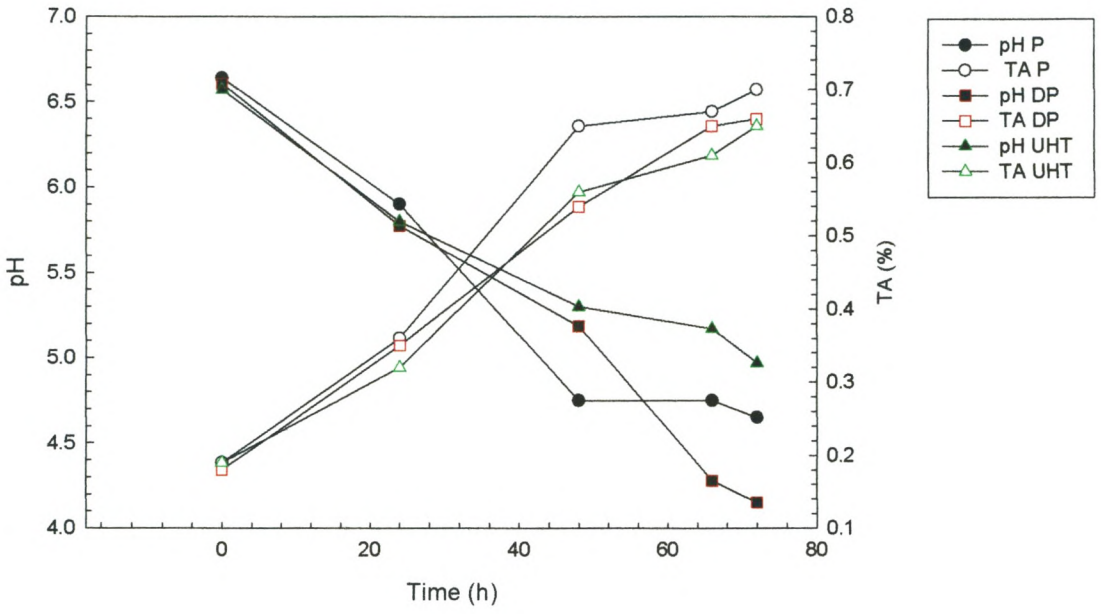

Figure 4. Changes in the pH and TA during the activation of mass-cultured Kefir grains and production of Kefir using Methods 9, with an inoculum size of 36 g grains.l^{-1} and different heat-treated milks (P = pasteurised; DP = double pasteurised, UHT = ultra high temperature).

Table 3. The means and *p*-values for the attributes of the sensory evaluation of Kefir produced by different grain:milk ratios and from different heat-treated milks.

Descriptor	Grain:milk ratio			*p*-value	Heat-treated milk			*p*-value
	36 g.l^{-1}	72 g.l^{-1}	108 g.l^{-1}		P	DP	UHT	
Appearance								
Smoothness 1	37.5	40.5	37.4	0.765924	46.3^{a}	68.5^{b}	76.3^{b}	0.000012
Thickness	59.3^{a}	63.9^{a}	45.0^{b}	0.040648	66.0	76.9	61.5	0.062350
Flavour								
Boiled milk	45.5^{a}	33.7^{ab}	23.2^{b}	0.003062	20.9^{a}	46.5^{b}	37.1^{ab}	0.002415
Yeasty	24.5^{a}	33.1^{a}	50.0^{b}	0.001150	22.5	17.0	19.4	0.504456
Sweetness	45.5^{a}	37.3^{ab}	27.7^{b}	0.014913	17.5^{a}	30.5^{b}	32.2^{b}	0.001704
Sourness	35.6^{a}	44.3^{a}	67.5^{b}	<0.000000	54.7^{a}	37.5^{b}	36.3^{b}	0.002443
Texture								
Prickly/Effervescence	23.4^{a}	32.8^{ab}	46.5^{b}	0.002146	30.2	19.0	19.2	0.0540†
Creamy mouth feel	57.0^{a}	46.1^{b}	36.3^{c}	0.0000301	47.6^{a}	63.6^{b}	68.0^{b}	0.000036
Smoothness 2	51.6^{a}	51.7^{a}	39.7^{b}	0.031697	55.0^{a}	75.0^{b}	82.9^{b}	0.000014
Chalky mouth feel	39.9	39.6	40.4	0.992437	39.7^{a}	32.2^{ab}	23.4^{b}	0.0497†
Acceptability								
Overall acceptability	57.0^{a}	59.3^{a}	35.0^{b}	0.000005	53.9	55.1	59.5	0.712383

Mean values marked with different letters are significantly different on a 95 % confidence level ($p \leq 0.05$).
† = *p*-values derived from Kruskal-Wallis non-parametric test
P = pasteurised milk
DP = double pasteurised milk
UHT = ultra high temperature milk

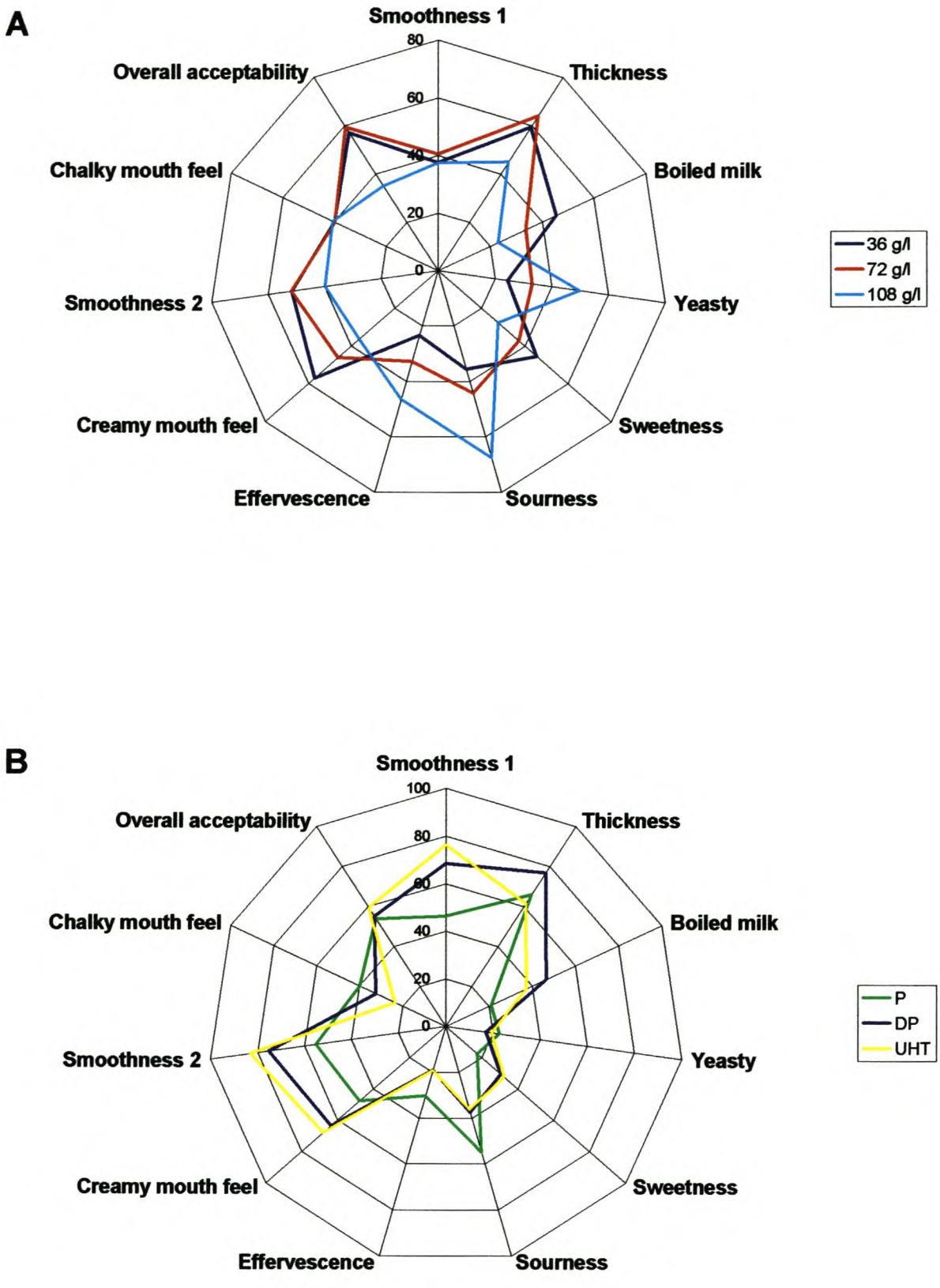

Figure 5. Radar plots of the sensory properties of Kefir produced with different inoculum sizes (**A**) and heat-treated milks (**B**).

present in Kefir grains are responsible for the production of carbon dioxide that gives the beverages its effervescent character (Duitschaever, 1989), therefore, it is to be expected that the beverage fermented with the higher yeast content and having the more yeasty flavour should be the most effervescent. The Kefir beverage produced with 108 g grains.l^{-1} did indeed have the highest perceived effervescence, although it only differed significantly from the Kefir prepared with 36 g grains.l^{-1}.

There was a significant difference with regards to the creamy mouth feel of the three beverages with the 36 g.l^{-1} beverage having the most creamy mouth feel and the 108 g.l^{-1} beverage the least. This is inversely related to the effervescence and a higher level of effervescence could lead to a decreased in creamy mouth feel.

With regard to overall acceptability, Kefir beverage produced with 108 g grains.l^{-1} was less acceptable than Kefir produced with 36 and 72 g grains.l^{-1}. These two beverages only differed significantly with regards to creamy mouth feel. The Kefir produced with 36 g grains.l^{-1} was distinguished as having a more creamy mouth feel. The two beverages proved to be very similar and they did not differ significantly with regards to overall acceptability. Therefore, Kefir made with an inoculum of 36 g grains.l^{-1} will have similar characteristics as Kefir produced from 72 g grains.l^{-1}. This was confirmed by the similar patterns in the radar plots (Fig. 5). Using less grains to produce Kefir has an economical advantage and an inoculum of 36 g grain.l^{-1} was thus used for further studies.

Sensory Study II - Comparison of sensory characteristics of Kefir prepared from different heat-treated milks.

Milk used for Kefir undergoes a heat treatment prior to fermentation to improve the product consistency by denaturing the whey proteins. This results in a good coagulum and better mouth feel (Marshall, 1993; Varnam & Sutherland, 1994; Brewer, 1998). Dairies often prefer to use UHT milk to manufacture Kefir (Marshall, 1993). The means and *p*-values of the sensory attributes evaluated are shown in Table 3 and the radar plot in Fig. 5. The summarised results of the analyses of variance are given in Table B5. The residual values for effervescence and chalky mouth feel did not conform to a norm curve and Kruskal-Wallis non-parametric tests were performed.

No significant differences were detected regarding yeasty flavour, effervescence and overall acceptability. Kefir produced from pasteurised milk (P) differed the most, while Kefir produced from double pasteurised (DP) and UHT milks were very similar. This could be because DP and UHT milk are similar due to the more severe heat treatment.

Kefir produced from P milk had a less smooth appearance and texture (more grits) and a less creamy mouth feel than the other two beverages. Heat-treatments more severe than normal pasteurisation of the milk lead to denaturing of the whey proteins, a good coagulum and better mouth feel (Marshall, 1993; Varnam & Sutherland, 1994; Brewer, 1998). Even though the *p*-value suggests that there is no significant difference in effervescence, the *p*-value is very low (0.0540). A box and whisker plot of the data shows that there is only a slight overlapping (Fig. B1). This would suggest that the effervescence of Kefir produced from P milk is higher. The beverage was also significantly sourer and less sweet than the other two beverages. Kefir produced from DP and UHT milk did not differ significantly with regards to any of the descriptors. This is confirmed by the similarity of the patterns on the radar plot (Fig. 5)

Conclusions

Kefir has the potential to be a successful fermented dairy product in the South African market since it has sensory characteristics comparable to that of the traditional fermented milk, Maas. The optimised method for the production of Kefir from mass-cultured grains was activation of the grains for two successive 24 h periods at 22°C, replacing the milk after each period, followed by production of Kefir at 22°C for 18 h. The fermentation vessel was swirled directly after inoculation and after 12 h of fermentation during activation and production of Kefir. The grains were removed from the fermented milk by sieving and the milk was incubated for a further 6 h at 18°C for a ripening period. This method resulted in a sour beverage with a thick consistency and the characteristic effervescence and flavour of Kefir. The optimised method also does not require the consumer to change the milk during the night. Taking the sensory characteristics and additional economical advantages into consideration, 36 g grains.l^{-1} was identified as the optimal inoculum size for the production of Kefir from mass-cultured grains.

Descriptive sensory analysis of Kefir prepared from different heat-treated milks indicated that Kefir prepared from double pasteurised and ultra high temperature (UHT) milks were very similar and had a better mouth feel than Kefir prepared from pasteurised milk. Therefore, it is recommended that the consumer purchase UHT milk for the production of Kefir as double pasteurisation requires additional preparation

Freezing, as a means to preserve Kefir grains, should be avoided or limited as it leads to a decrease in the mass and acidification activity of the grains. The majority of the target market (low-income consumers) does not own freezing facilities and another method of preservation needs to be considered. Freeze-drying of mass-cultured grains should be investigated as an alternative method of preservation and the production of Kefir from freeze-dried mass-cultured grains must be optimised.

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APPENDIX A

To Chapter Three

Panellist: _____ Date: _____

Session number: _____

A. APPEARANCE

1. Smoothness

gritty — extremely smooth

2. Thickness

runny — drinking yoghurt

B. FLAVOUR

3. Boiled milk

none — extreme

4. Yeasty

none — extreme

5. Sweetness

none — extreme

6. Sourness

none — extreme

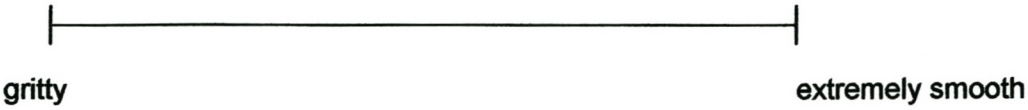
Figure A1. Sensory evaluation form for the quantitative descriptive analysis of Kefir.

C. TEXTURE

7. Effervescence / prickly (CO_2)

8. Creamy mouth feel

9. Smoothness



10. Chalky mouth feel

D. ACCEPTABILITY

11. Overall acceptability

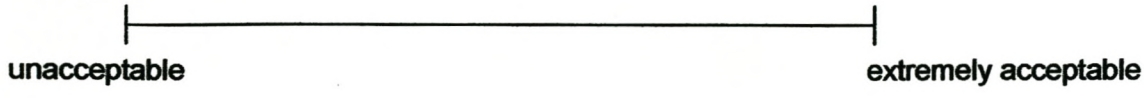


Figure A1. Cont.

SENSORY EVALUATION OF KEFIR **Date:**_____

Name of panel member:_____

Session nr._____

Instructions

- Evaluate the samples from left to right
- Refresh mouth between each sample with water and water biscuit

A	B	C
CODE	CODE	CODE

Figure A2. Sample placement form.

APPENDIX B

To Chapter Three

Data are given in this appendix to simplify the discussion section of this chapter.

Table B1. Changes in the mass of Kefir grains when activated and used to produce Kefir using Methods 1 - 3.

Method 1		Method 2		Method 3	
Mass of grains (g)	Time (h)	Mass of grains (g)	Time (h)	Mass of grains (g)	Time (h)
17.98	0	18.049	0	18.195	0
13.7	18	11.66	18	12.62	18
11.45	36	11.104	36	11.788	36
11.95	54	9.584	54	11.606	52
12.66	72	8.582	72	11.402	70
12.43	88	8.161	90	11.373	86
		8.361	108	12.038	104
69.13*		46.32*		66.16*	

*End mass as a percentage of the original mass (m/m)

Table B2. Changes in the mass of Kefir grains when activated and used to produce Kefir using Method 4 with inoculums of 72 and 144 g grains.l^{-1} (in duplicate).

Time (h)	Mass of grains (g)			
	72g.l^{-1}		144g.l^{-1}	
0	17.9	18.158	35.86	36
18	18.02	20.34	33.32	34.36
36	18.08	17.06	33.03	34.05
54	17.05	18.56	34.03	33.8
72	17.58	19.06	34	31.75
90	18.7	20.79	33.52	32.12
	104.47*	114.49*	93.47*	89.22*

*End mass as a percentage of the original mass (m/m)

Table B3. Changes in the mass of previously frozen and fresh mass-cultured Kefir grains when activated and used to produce Kefir using Method 9, with an inoculum of 36 g grains.l^{-1} (in duplicate).

Time (h)	Mass of grains (g)			
	Frozen grains		Fresh grains	
0	18.036	18.249	17.9275	18.6465
24	10.8736	11.585	25.55	25.591
48	8.3551	9.19	27.5679	28.234
66	6.7082	7.775	32.987	31.2792
	37.19*	42.61*	184.00*	167.75*

*End mass as a percentage of the original mass (m/m)

Table B4. Summary of the analyses of variance for sensory characteristics of Kefir produced from pasteurised, double pasteurised and UHT milk.

	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Smoothness 1	130.00	2	64.998	14559.29	60	242.6549	0.26786	0.765924
Thickness	3908.53	2	1954.263	33471.59	58	577.0963	3.38637	0.040648
Boiled milk	5225.44	2	2612.722	24552.43	60	409.2071	6.38484	0.003062
Yeasty	7042.83	2	3521.414	27828.53	60	463.8088	7.59238	0.001150
Sweetness	3321.48	2	1660.738	22071.71	60	367.8618	4.51457	0.014913
Sourness	11431.59	2	5715.797	18147.72	60	302.4620	18.89757	0.000000
Effervescence	5674.69	2	2837.346	24968.12	60	416.1354	6.81832	0.002146
Creamy mouth feel	4482.10	2	2241.051	14441.04	60	240.6840	9.31118	0.000301
Smoothness 2	1984.22	2	992.108	16273.25	60	271.2208	3.65793	0.031697
Chalky mouth feel	7.40	2	3.698	29224.69	60	487.0782	0.00759	0.992437
Overall acceptability	7569.94	2	3784.972	15084.97	60	251.4162	15.05461	0.000005

Table B5. Summary of the analyses of variance for sensory characteristics of Kefir produced from pasteurised, double pasteurised and UHT milk.

	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Smoothness 1	11.656.58	2	5828.292	30065.42	69	435.7307	13.37590	0.000012
Thickness	30.1.78	2	1508.389	36017.83	69	521.9976	2.88965	0.062350
Boiled milk	8068.75	2	4034.375	42279.25	69	612.7428	6.58413	0.002415
Yeasty	376.33	2	188.167	18786.54	69	272.2687	0.69111	0.504456
Sweetness	3117	2	1558.500	15358.50	69	222.5870	7.00176	0.001704
Sourness	5047.37	2	2523.686	26083.73	68	383.5842	6.57922	0.002443
Effervescence	1951.03	2	975.514	19220.25	69	278.5543	3.50206	0.035597
Creamy mouth feel	5522.25	2	2761.125	15987.25	69	231.6993	11.91685	0.000036
Smoothness 2	9904.19	2	4952.097	25790.79	69	373.7796	13.24871	0.000014
Chalky mouth feel	3190.86	2	1595.431	35354.13	69	512.3786	3.11377	0.050733
Overall acceptability	414.19	2	207.097	41928.42	69	607.6582	0.34081	0.712383

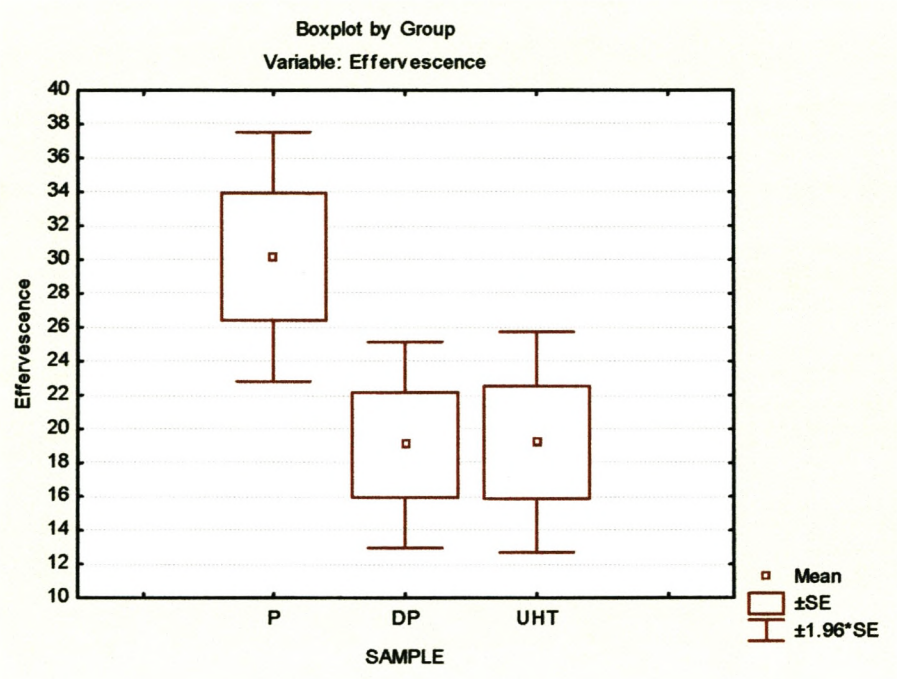


Figure B1. Box and whiskers plot for the descriptor Effervescence of Kefir produced by P, DP and UHT milk.

CHAPTER 4

OPTIMISATION OF THE PRODUCTION OF KEFIR BEVERAGE FROM FREEZE-DRIED MASS-CULTURED GRAINS

Abstract

Freeze-drying is a method that can be applied to successfully preserve Kefir grains for long-term storage and it provides a form of Kefir grain that can be easily distributed. The aim of this study was to investigate the time required to freeze-dry and rehydrate South African mass-cultured Kefir grains and to optimise the production of the Kefir beverage from these grains. The chemical compositions of mass-cultured grains (MC), mass-cultured, freeze-dried grains (MCFD), mass-cultured, freeze-dried grains that have been rehydrated and activated (FDRA) and activated mass-cultured grains that have been freeze-dried and rehydrated (AFDR) were also investigated. The minimum period for freeze-drying of grains was identified as 2 d and the optimal rehydration time of freeze-dried grains in tap water was found to be 1 h. Kefir beverages prepared from freeze-dried activated (FDRA) grains and activated freeze-dried grains (AFDR) did not differ significantly with regards to the sensory attributes examined. They were found to be less viscous, less sour and had a sweeter and more boiled milk flavour than Kefir beverage produced from mass-cultured grains, indicating that the freeze-dried grains were less active. Kefir beverages produced from freeze-dried grains were less acceptable than Kefir produced from mass-cultured grains.

Introduction

Kefir is a refreshing fermented milk beverage with a sour and distinctly yeasty taste, resulting from the fermentation of milk with Kefir grains (Saloff-Coste, 1996; Roginski, 1988). The grains are gelatinous granules consisting of a complex combination of microbes embedded in a polysaccharide matrix called kefiran and can be recovered and reused after fermentation (Özer & Özer, 2000; Saloff-Coste, 1996; Marshall, 1993; La Rivière *et al.*, 1967).

The Kefir beverage is commercialised in various parts of the world, but problems are encountered due to secondary fermentation in the beverage that

leads to the formation of carbon dioxide and blown packages (Özer & Özer, 2000; Kwak *et al.*, 1996). Problems are also experienced in producing a product with constant characteristics due to the varying microbial activity of the grains (Kemp, 1984). Therefore, it might be more advantageous to market the Kefir grains themselves, which would allow the consumer to produce their own fresh Kefir beverage at home.

In order to commercialise Kefir grains, it will be necessary to be able to produce large quantities of grains by continuous fermentation in milk, during which they increase in size and number (Marshall & Cole, 1985). Schoevers & Britz (2003) investigated the influence of different culturing conditions on the Kefir grain biomass increase and then developed a method for the rapid production of mass-cultured Kefir grains.

Freeze-drying was identified as the most effective method to preserve Kefir grains for long-term storage and the dried grains would then facilitate the distribution (Brialy *et al.*, 1995; Cilliers, 2001). Freeze-drying allows the microbes to be preserved during and after drying (Pebley & Baglien, 2003). Freeze-dried grains are commercially available in various countries and have been shown to retain activity at room temperature for 12 - 18 months (Oberman & Libudzisz; 1998). However, more than 80% of the yeasts are lost during freeze-drying and these grains are often supplemented by the addition of yeast isolates.

The sensory characteristics of the Kefir beverage produced from freeze-dried mass-cultured grains have not been studied. In addition, freeze-drying is an expensive form of dehydration because of the slow drying rate and the use of expensive equipment (Liapis & Bruttini, 1995). Therefore, it is necessary to determine the minimum time required to freeze-dry mass-cultured Kefir grains, which will still provide adequate preservation. The aim of this study was to optimise the production of Kefir from mass-cultured freeze-dried grains to produce an acceptable Kefir beverage. The influence of different freeze-drying and rehydration periods of the Kefir grains was also investigated and the chemical composition of mass-cultured and freeze-dried grains with regards to protein, fat, moisture, ash and carbohydrates was determined.

Materials and methods

Starter culture

Frozen mass-cultured Kefir grains were obtained from the University of Stellenbosch Food Science Culture Collection and used for mass-culturing according to the method developed by Schoevers & Britz (2003). The mass-cultured grains were stored at -18°C for no longer than 3 months.

Freeze-drying and rehydration of mass-cultured Kefir grains

Approximately 26 g mass-cultured grains were placed in sterile glass petridishes and frozen at -18°C for 24 h. The frozen grains were freeze-dried under constant vacuum at -20°C (Virtis Preservator Model 10-PR) for periods of 1, 2, 3 and 6 d to determine the changes in mass during the different freeze-drying periods. Four replications were done for each freeze-drying period.

The minimum rehydration time for optimum moisture absorption was determined by covering 2 g freeze-dried mass-cultured grains with 45 ml of water. The grains (triplicate units) were allowed to absorb the water for 1, 2, 6, 12, 16 and 18 h. After rehydration, the excess moisture was removed by sieving through a sterile kitchen sieve and the mass of the rehydrated grains was determined. The percentage rehydration was calculated by the difference between the rehydrated and original mass as a percentage of the original mass.

Chemical analysis of Kefir grains

The moisture, ash, crude protein, fat and total carbohydrate contents of fresh mass-cultured grains (MC), mass-cultured and then freeze-dried grains (MCFD), freeze-dried mass-cultured grains that were rehydrated and activated (FDRA) and activated mass-cultured grains that were freeze-dried and then rehydrated (AFDR), were determined. Rehydration of the grains was carried out for 4 h. The grains, excluding the MCFD grains, were rinsed with sterile water, dried on paper towels and all the grains were finely ground with a mortar and pestle prior to chemical analysis.

The moisture content of triplicate samples of each treatment was determined by the method of James (1996). Approximately 5 g of the sample was placed in a pre-dried aluminium moisture dish. The samples were pre-dried for 30

min over a steambath prior to drying at 70°C for 18 h under constant vacuum. The samples were allowed to cool in a desiccator, weighed and the percentage moisture (m/m) was calculated. The total solids content was calculated by subtracting the moisture content from the total sample weight (Bradley, 2003).

The ash content of triplicate samples was determined by the method of James (1996). Five gram of the sample was placed in a pre-dried porcelain crucible, 5 ml of magnesium acetate alcohol was poured over the samples and the crucibles were then heated over a Bunsen burner until the samples turned black. The crucibles were placed in a muffle furnace and incinerated for 18 h at 550°C, cooled in a desiccator, weighed and the ash content calculated.

The protein content of quadruplicate grain samples of each treatment was determined with the Dumas combustion method using a Leco FP528 Nitrogen Analyzer, following standard AOAC (1990) procedures. The fat content of triplicate samples was determined with the AOAC (1990) method, which consists of a hydrolysis with HCl followed by an ether extraction. The carbohydrate content was calculated by subtracting the ash, protein and fat percentages from the total solids (Wszolek *et al.*, 2001).

Sensory evaluation of Kefir produced with fresh mass-cultured and freeze-dried grains

Sensory analysis was performed on three Kefir beverage samples: Kefir beverage produced from activated fresh mass-cultured grains (MC); Kefir produced from mass-cultured grains that were freeze-dried, rehydrated and activated (FDRA); and Kefir produced from activated, freeze-dried and rehydrated mass-cultured grains (AFDR). Pasteurised milk from a local supermarket was heat-treated (double pasteurisation) in a temperature-controlled waterbath at 85° ± 2°C for 20 min. The double pasteurised milk (500 ml) was transferred to 1 litre sterile containers and allowed to cool to room temperature. The double pasteurised milk was used for both the activation of the Kefir grains and the production of the Kefir beverage.

To prepare the MC Kefir beverage, 500 ml of double pasteurised milk was inoculated with 18 g of freshly mass-cultured grains and the grains were activated by incubation for two successive 24 h incubation periods at 22°C. The grains were recovered from the fermented milk between every incubation period by sieving

through a sterile kitchen sieve and were then inoculated into fresh double pasteurised milk. Directly after each inoculation, the fermentation vessel was swirled five times and again after 12 h of incubation. The activated grains were then used to make Kefir beverage by inoculation into 500 ml double pasteurised milk and incubating the mixture at 22°C for 18 h. The grains were then removed and the fermented milk was incubated for a "maturation period" of 6 h at 18°C.

FDRA Kefir beverage was prepared by freeze-drying mass-cultured grains for 2 d, followed by rehydration in water for 2 h as previously described. The rehydrated grains were activated and Kefir beverage prepared by the same procedure as for MC. For AFDR Kefir, the grains were prepared as for MC and then freeze-dried for 2 d. The freeze-dried grains were rehydrated for 2 h in water and 18 g of rehydrated grains were inoculated in 500 ml double pasteurised milk. The inoculated milk was fermented at 22°C for 24 h, where after the grains were sieved out and inoculated in 500 ml fresh double pasteurised fermented milk. The inoculated milk was fermented at 22°C for 18 h, after which the grains were sieved out and the fermented milk was incubated at 18°C for a further 6 h to mature. The prepared Kefir beverages were refrigerated overnight (± 18 h) before sensory analyses were performed.

Kefir beverage samples were evaluated using quantitative descriptive analysis (QDA) (Stone *et al.*, 1974). Seven panellists were recruited from staff and post-graduate students from the Department of Food Science at the Stellenbosch University and trained. Experimental vocabulary was developed during the initial training sessions by modification of the vocabulary used in similar sensory analyses of Kefir and Maas (Muir *et al.*, 1999; Wszolek *et al.*, 2001; Human, 1998), and suggestions from the panel members. The descriptors were classified into four groups namely appearance, taste, texture and acceptability and are listed in Table 1. Perceived visual smoothness designated "Smoothness 1" refers to visually evaluated smoothness and "Smoothness 2" refers to orally perceived smoothness. The sensory evaluation and sample placement forms used are given in Appendix A at the end of the previous chapter, Chapter 3. The intensity ratings were scored on a 10 cm unipolar unstructured line scale, with verbal anchors at either side. Panellists were asked to place a vertical line across the horizontal line at a point that best quantified their perception of intensity during the evaluation of the samples, which was then converted to a scale of 0 to 100. Sensory evaluation

Table 1. Descriptors used for the sensory evaluation of Kefir.

Descriptor	Scale (0 →100)	Definition
Appearance		
Smoothness 1	gritty → extremely smooth	Grittiness on the back of a metal spoon dipped in the sample
Thickness	runny → drinking yoghurt	Degree of thickness measured by spooning the sample
Flavour		
Boiled milk	none → extreme	Flavour of boiled milk
Yeasty	none → extreme	Yeasty flavour
Sweetness	none → extreme	Sweet taste
Sourness	none → extreme	Sour taste
Texture		
Prickly/Effervescence	none → extreme	Prickly feeling on the tongue
Creamy mouth feel	watery → extremely creamy	Degree of creamy mouth coating
Smoothness 2	gritty → extremely smooth	Perceived smoothness of the sample in the mouth
Chalky mouth feel	none → extreme	Floury-dry after-taste
Acceptability		
Overall acceptability	unacceptable → extremely acceptable	Perceived liking of the sample

was carried out under fluorescent lighting conditions in an adequately ventilated area. Approximately 50 ml of each sample was presented in 125 ml white styrofoam cups covered with foil and stainless steel teaspoons were provided to assist in the evaluation. Each sample was assigned a random three-digit code and samples were presented in a random order regarding replicate and panellist during four consecutive tasting sessions with half hour breaks in between. Panellists were asked to refresh their palates between every sample with distilled water and table water biscuits.

The pH and percentage titratable acidity (TA) of the initial milk and of the fermented milk were determined following every incubation period duing the preparation of the Kefir beverages. The pH was measured with a Mettler Toledo 320 pH meter and the TA was determined in duplicate by titration of a 9 ml sample with 0.1 N NaOH to the faint pink phenolphthalein endpoint (Dixon, 1973).

Statistical analysis

A one-way analysis of variance (ANOVA) was performed for the sensory study using Statistica™ 6 (version 6.1.409) for Windows™, the sources of variance being different types of grains (MC, AFDR and FDRA). A *p*-value smaller or equal to 0.05 was considered as an indication of significant difference between mean values. If the samples differed significantly an *ad hoc* test, Bonferroni, was performed to determine which samples differed. In the case of the residual values not conforming to a norm curve a non-parametric test, Kruskal-Wallis, was performed. A radar plot was drawn using Excel™ 2000 (version 9.0.2720) for Windows™.

Results and discussion

Freeze-drying and rehydration of mass-cultured Kefir grains

Mass-cultured Kefir grains were freeze-dried for different time periods to determine the shortest period required as freeze-drying is expensive. The results are represented in Table 2. A significant difference was found between grains that were freeze-dried for 1 and 6 d, with a *p*-value of 0.029. Two days (48 h) was identified as the minimum period required to freeze-dry mass-cultured grains.

The degree of rehydration of freeze-dried grains after 1, 2, 6, 12, 16 and 18 h is presented in Fig. 1. An analysis of variance resulted in a *p*-value of 0.055 that indicates no significant difference between the rehydration times. However, the *p*-value may still be regarded as meaningful as it is close to 0.05 and a repeat of the experiment with three or more replications might lead to a significant difference. Rehydration for 1 h resulted in a higher rehydration percentage than longer rehydration times and is thus recommended as the optimal time. It was found that after 1 h, the grains start to break up in the water and are lost when the excess water is removed by sieving.

Chemical analysis of Kefir grains

Mass-cultured Kefir grains (MC), mass-cultured and then freeze-dried Kefir grains (MCFD), freeze-dried mass-cultured grains that were rehydrated and activated (FDRA), and activated mass-cultured grains that were freeze-dried and then rehydrated (AFDR), were chemically analysed for ash, protein, fat, moisture and carbohydrate content and the data are depicted in Table 3. The carbohydrate values were calculated as 4.24, 29.64, 5.05 and 3.17% for MC, MCFD, FDA and AFDR respectively. The composition of MCFD grains differed significantly from the other treated grains with regards to all the measured parameters as they are in a dry state and could, therefore, be considered as "concentrated".

It has been shown in the literature that the chemical composition of Kefir grains varies depending on their origin. Wet grains originating from Russia had a lower ash content (0.7%), higher moisture content (90%), lower protein content (3.2%) and a lower fat content (0.3%) (Ottogalli *et al.*, 1973) than the wet grains analysed in this study. Kefir grains that originated from Argentina were found to comprise of similar moisture contents (79 - 83%), lower protein contents (4.7 - 6.6%) and the same carbohydrate contents (4.3 - 5.4%) as the wet grains analysed in this study (Garrote *et al.*, 2001). Therefore, the grains analysed in this study had a composition comparable to that of grains originating from Argentina.

Sensory Evaluation of Kefir beverage produced with mass-cultured and freeze-dried grains

Kefir beverage was produced from activated mass-cultured grains (MC),

Table 2. Average decrease in mass (for four repeats) of Kefir grains freeze-dried for different periods.

Time (d)	Mass decrease ± Stdev (%)
1	76.65 ± 4.63^{a}
2	81.90 ± 0.32^{ab}
3	83.33 ± 2.18^{ab}
6	83.44 ± 2.25^{b}

Mean values marked with different letters are significantly different on a 95% confidence level ($p \leq 0.05$). Stdev = standard deviation.

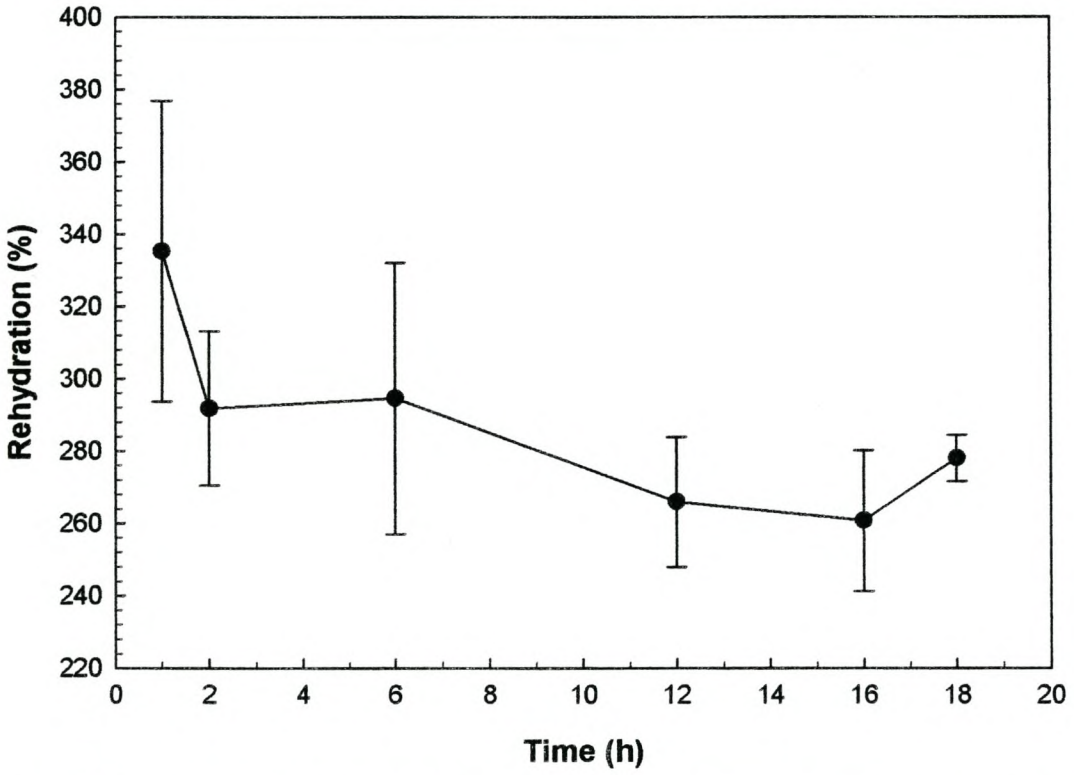


Figure 1. Average increase in mass (of triplicate samples) of Kefir grains rehydrated for different time periods. Error bars represent the standard deviation.

Table 3. Mean percentages (triplicate samples) and standard deviations of ash, moisture, fat and protein content of Kefir grains.

	MC	MCFD	AFDR	FDRA
Ash	1.77 ± 0.20^{a}	6.69 ± 0.07^{b}	1.10 ± 0.02^{c}	1.63 ± 0.31^{ac}
Moisture	82.81 ± 0.15^{a}	4.70 ± 0.06^{b}	81.95 ± 0.11^{c}	83.59 ± 0.28^{d}
Fat	1.35 ± 0.05^{a}	13.93 ± 0.62^{b}	3.24 ± 0.08^{c}	2.42 ± 0.17^{c}
Protein	9.83 ± 0.02^{a}	45.03 ± 0.65^{b}	10.54 ± 0.09^{a}	7.32 ± 0.09^{c}

Mean values marked with different letters in one row are significantly different on a 95% confidence level ($p \leq 0.05$).

MC = mass-cultured

MCFD = mass-cultured, freeze-dried

AFDR = activated mass-cultured grains that were freeze-dried and rehydrated

FDRA = freeze-dried mass-cultured grains, rehydrated, activated

freeze-dried mass-cultured grains that were rehydrated and activated (FDRA), and mass-cultured activated grains that were freeze-dried and rehydrated (AFDR). The Kefir beverages were evaluated by a trained sensory panel to characterise the differences in sensory properties.

The means and *p*-values of the sensory attributes evaluated are shown in Table 4 and the radar plot in Fig. 2. The summarised results of the analyses of variance are in Table B1 in the appendix of this chapter. The residual values for the descriptors sweetness and sourness did not conform to a norm curve and Kruskal-Wallis non-parametric tests were performed.

The samples did not differ significantly with regards to smoothness 1 and 2, yeasty, effervescence and chalky mouth feel. The Kefir beverages produced from the freeze-dried grains (FDRA and AFDR) did not differ significantly from each other regarding any of the descriptors (Table 4) and their radar plots are almost identical (Fig. 2). The MC Kefir beverage was thicker and had less boiled milk flavour and sweetness than FDRA and AFDR Kefir. These significant differences indicate that the freeze- dried grains were less active than the fresh mass-cultured grains. Grains that have a higher acidification activity will produce Kefir that is less sweet and more sour with a thicker curd. It will be more fermented, leading to a decrease in the boiled milk flavour that is a dominant flavour of the double pasteurised milk used as fermentation medium. MC Kefir beverage had a creamier mouth feel than the FDRA, was more sour than the AFDR and was perceived as being more acceptable than the FDRA and AFDR.

Changes in pH and TA during production of Kefir

The pH and TA values were measured during the activation of the grains and the production of Kefir for the sensory evaluation (Fig. 3). The final pH values for MC, FDRA and AFDR Kefir were 4.24, 4.64 and 4.64, respectively and the final TA values were 0.72, 0.66 and 0.73%, respectively. This confirms the conclusion of the sensory analysis, namely that the grains used to produce MC Kefir were more active than the grains used to produce FDRA and AFDR as the pH is lower and the TA higher.

Table 4. The means and p-values for the attributes of the sensory evaluation of Kefir produced from MC, FDRA and AFDR grains.

Descriptor	MC	FDRA	AFDR	p-value
Appearance				
Smoothness 1	52.5	43.5	48.1	0.372
Thickness	59.0[a]	36.4[b]	35.9[b]	$p \leq 0.001$
Flavour				
Boiled milk	19.1[a]	38.5[b]	41.0[b]	0.002
Yeasty	18.4	24.1	26.8	0.350
Sweetness	27.5[a]	43.2[b]	43.7[b]	0.007†
Sourness	40.6[a]	28.0[ab]	24.9[b]	0.020†
Texture				
Prickly/Effervescence	14.3	16.6	18.5	0.647
Creamy mouth feel	57.2[a]	44.5[b]	45.8[ab]	0.007†
Smoothness 2	63.8	57.0	57.4	0.414
Chalky mouth feel	32.3	31.5	35.9	0.675
Acceptability				
Overall acceptability	50.5[a]	27.4[b]	32.1[b]	$p \leq 0.001$

Mean values marked with different letters are significantly different on a 95% confidence level ($p \leq 0.05$).

† = *p*-values derived from Kruskal-Wallis non-parametric tests

MC = Kefir produced from mass-cultured grains

FDRA = Kefir produced from mass-cultured grains that were freeze-dried, rehydrated and activated

AFDR = Kefir produced from mass-cultured grains that were activated, freeze-dried and rehydrated

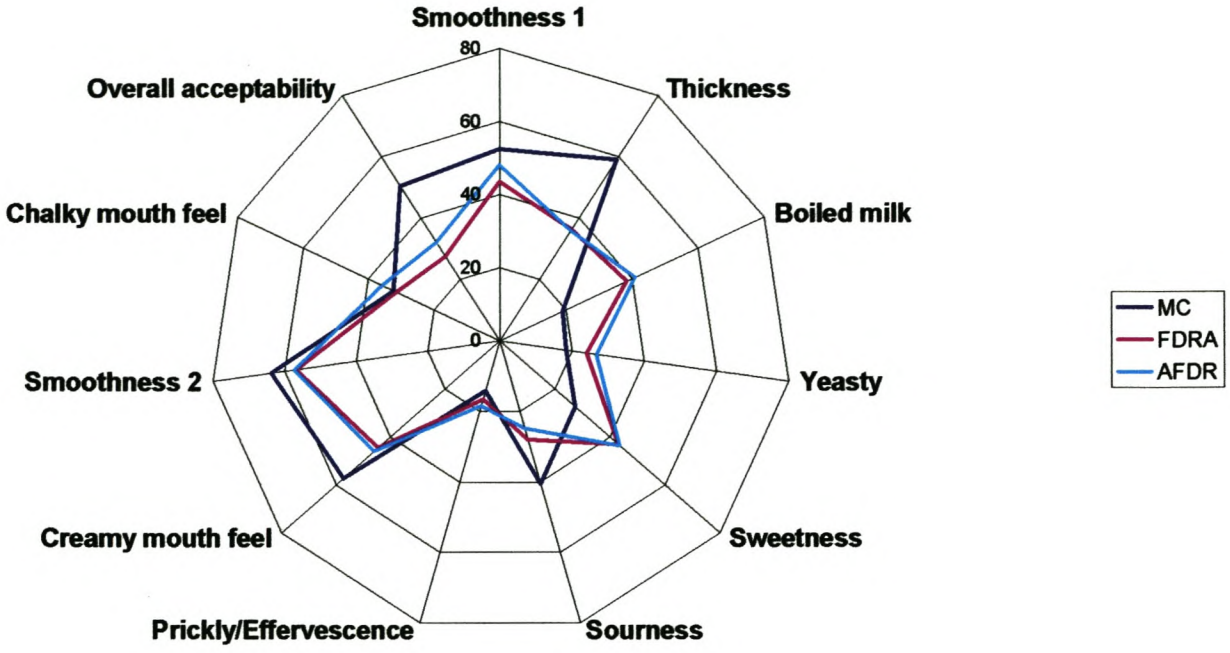


Figure 2. Radar plots for the sensory characteristics of Kefir produced from mass cultured grains (MC), mass-cultured grains that were freeze-dried, rehydrated and activated (FDRA) and mass-cultured grains that were activated, freeze-dried and rehydrated (AFDR).

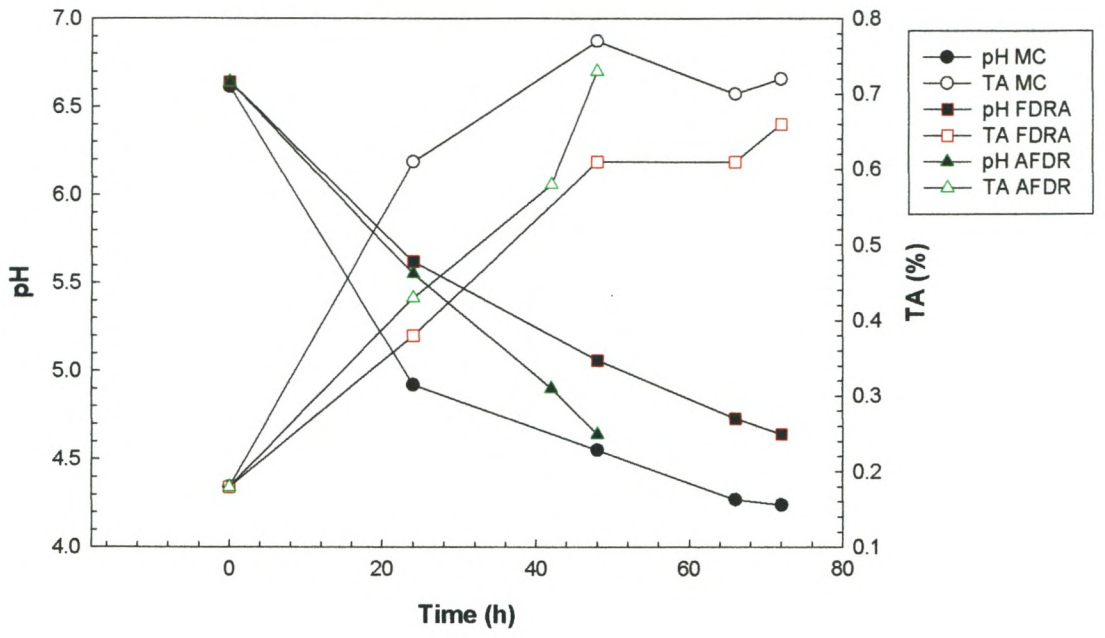


Figure 3. Changes in the pH and TA during the activation of mass cultured grains (MC), mass-cultured grains that were freeze-dried and rehydrated and activated (FDRA) and mass-cultured grains that were activated, freeze-dried and rehydrated (AFDR) and the production of Kefir beverage.

Conclusions

Freeze-drying can be applied successfully as a method to preserve Kefir grains and freeze-dried Kefir grains are commercially available in numerous countries. Mass-cultured Kefir grains must be freeze-dried for a period of 2 days for sufficient drying, considering the economical complications of the process. The consumer who purchases the freeze-dried grains can easily rehydrate the grains by placing them in tap water for 1 h, where after the grains can be activated in milk and used to produce Kefir beverage.

Kefir produced with activated freeze-dried mass-cultured grains and freeze-dried activated mass-cultured grains had similar sensory characteristics and were less acceptable than Kefir produced with mass-cultured grains. The freeze-dried grains were less active than the mass-cultured grains and resulted in beverages that were not adequately fermented. For commercialisation purposes, it is recommended that the grains are activated and then freeze-dried. This would be of convenience to the consumer as additional activation would not be required. Future research needs to be done to investigate the effect of larger inoculum sizes of freeze-dried grains on the sensory characteristics of Kefir.

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APPENDIX A

To Chapter Four

Data are given in this appendix to simplify the discussion section of this chapter.

Table B1. Summary of the analyses of variance for the sensory characteristics of Kefir produced from MC, FDRA and AFDR Kefir grains.

	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Smoothness 1	963.08	2	481.542	33113.79	69	479.9100	1.00340	0.371917
Thickness	8329.08	2	4164.542	22724.42	69	329.3394	12.64514	0.000021
Boiled milk	6897.53	2	3448.764	33303.79	69	482.6636	7.14527	0.001513
Yeasty	886.08	2	443.042	28715.42	69	416.1655	1.06458	0.350468
Sweetness	4077.69	2	2038.847	34312.92	69	497.2886	4.09993	0.020774
Sourness	3331.44	2	1665.722	31291.21	69	453.4958	3.67307	0.030489
Effervescence	213.08	2	106.542	16754.79	69	242.8231	0.43876	0.646620
Creamy mouth feel	2364.19	2	1182.097	14868.42	69	215.4843	5.48577	0.006152
Smoothness 2	682.86	2	341.431	26389.75	69	382.4601	0.89272	0.414216
Chalky mouth feel	268.11	2	134.056	23422.33	69	339.4541	0.39492	0.675250
Overall acceptability	7141.19	2	3570.597	12848.46	69	186.2095	19.17516	0.000000

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The consumption of dairy products is low among the black population in South Africa due to the high occurrence of lactose intolerance (MacIntyre *et al.*, 2002). Fermented milk products can be consumed by most lactose intolerant individuals, of which traditional Maas is the most popular (Keller & Jordaan, 1990). Due to legislation restricting the sale of unpasteurised milk, the low-income Black urban communities cannot make traditional Maas and commercial Maas is expensive and a poor equivalent (Anon., 1997; Berry, 1999). Kefir is a fermented dairy product with sensory characteristics comparable to that of traditional Maas, is low-cost, nutritious and safe to consume (Van Wyk *et al.*, 2002).

Kefir is a self-carbonated, fermented dairy beverage made with reusable Kefir grains (Steinkraus, 1996; Özer & Özer, 2000). The grains are stable conglomerates of lactic acid bacteria and yeasts imbedded in and held together by kefiran, an insoluble polysaccharide material (Steinkraus, 1996). Due to various factors, it will be preferable to commercialise the Kefir grains itself in South Africa for household production of Kefir. Kefir grains can be mass-cultured and preserved by freeze-drying. Both these processes have an influence on the microbial balance of the grains and, therefore, on the sensory characteristics of the Kefir produced from the grains (Kuo & Lin, 1999; Liu *et al.*, 1999; Witthuhn *et al.*, 2004). The aim of this study was to optimise the production of Kefir from South African mass-cultured grains and from mass-cultured, freeze-dried grains.

The optimised method developed during this study for the production of Kefir from mass-cultured South African Kefir grains consists of two periods of Kefir grains activation at 22°C for 24 h each, followed by Kefir production at 22°C for 18 h. The grains were then sieved out and the fermented milk was incubated at 18°C for 6 h to mature. During activation and Kefir production the fermentation vessel was swirled five times after inoculation and again after 18 h during every incubation period. This optimised method resulted in a sour beverage with a thick consistency and the characteristic flavour and effervescence of Kefir. An inoculum size of 36 g grains.l^{-1} was identified as the optimal inoculum size.

Production of Kefir from mass-cultured grains with the optimised method and 36 g grains.l^{-1} using different heat-treated milks (pasteurised, double pasteurised and UHT) showed that the Kefir beverages produced from double pasteurised and UHT milk were similar and superior in sensory characteristics to Kefir produced from pasteurised milk.

It was found that a liquid residue forms on the defrosting of Kefir grains and that mass loss of Kefir grains occurs upon defrosting and activation of Kefir grains that were stored at -18°C. Therefore, frozen storage is not the optimal method to preserve Kefir grains and the freeze-drying of mass-cultured grains were investigated. Since freeze-drying is an expensive process, the minimum time required to freeze-dry Kefir grains was investigated and was found to be 2 d. The freeze-dried grains were then rehydrated in tap water for different times and 1 h of rehydration was found to be optimal.

Kefir produced with activated freeze-dried mass-cultured grains and freeze-dried activated mass-cultured grains had similar sensory characteristics. Both beverages were less acceptable than Kefir produced with mass-cultured grains and showed properties that indicated that they were less fermented, suggesting that the freeze-dried grains were less active than the fresh mass-cultured grains. The activity of the freeze-dried grains will, however, increase with subsequent production of Kefir. For the purpose of commercialisation, it is recommended that the grains are activated and then freeze-dried. This would render the activation of the rehydrated grains unnecessary adding to consumer convenience.

The influence of larger grain:milk ratios of freeze-dried grains and longer activation periods on the sensory characteristics of freeze-dried grains needs to be investigated. Future research must also be done on the supplementation of freeze-dried grains with lactic acid bacteria and yeast isolates to improve the sensory characteristics and activity of freeze-dried Kefir grains. Future sensory research should be done to determine the preference of the consumer with regards to grain:milk ratio, heat-treated milk and freeze-dried grains by performing a sensory analysis with a consumer panel consisting of individuals from the target market.

Kefir is being marketed successfully in various parts of the world. The beverage has various health benefits and an inhibitory activity towards certain pathogens and spoilage organisms (Saloff-Coste, 1996; Garrote *et al.*, 2000).

Kefir also has a taste comparable to that of traditional Maas and is a very appropriate and necessary product for the South African market (Van Wyk *et al*, 2002).

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NUWE USVV KOMITEE VERKIESING *1 OKTOBER 2004*

Die nuwe USVV komitee vir die 2004/2005 termyn is deur die nagraadse studente verkies.

TWEEDEJAARS PIZZADAG *7 OKTOBER 2004*

Die tweedejaar Voedselwetenskap studente is met pizzas bederf tydens 'n klasperiode. Die pizzas is van Roman's Pizzas bestel en alle uitgawes is deur 'n gedeelte van die studente se USVV ledegeld gedek. Hierdie was ook die laaste geleentheid wat gereël is deur die 2003/2004 USVV komitee.